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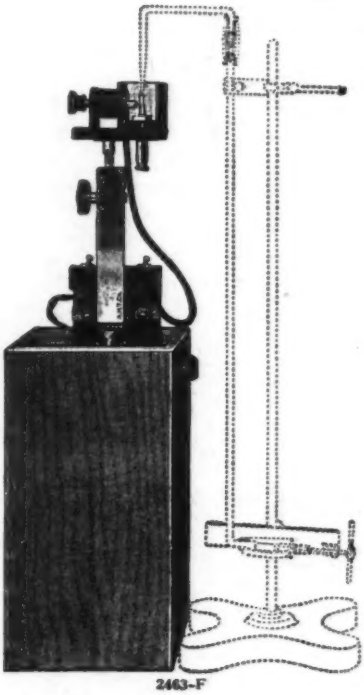

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AN ANALYSIS OF THE MOLTING PROCESS IN THE FIDDLER CRAB, UCA PUGILATOR [1]

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The spectacular manner by which decapod crustaceans accomplish growth through molting has fascinated biologists for a long time. Considerable information has been accumulated during the past twenty years with respect to physiological changes which accompany molt. One of the results of these investigations has been the establishment of an hypothesis that hormones act as regulatory factors of certain processes associated with growth and molt.

Koller (1930) was the first to demonstrate a difference between normal and eyestalkless crustaceans in the inorganic content of exuviae; Koller used the shrimp, *Crangon* (*Crago*) *vulgaris*. Plankemann (1935) made similar observations for some other crustaceans. The first experimental demonstration that the sinus gland was involved in the regulation of molt was made for the crayfish, *Cambarus*, by Brown and Cunningham (1939); these investigators showed that acceleration of molt resulted from eyestalk removal, and that following eyestalk removal, the accelerated molting was retarded by sinus gland implants. Abramowitz and Abramowitz (1940) and Kleinholz and Bourquin (1941a) noted that eyestalk ablation in *Uca pugilator* shortened the intermolt period, but these investigators did not interpret their results as a demonstration of hormonal control of molt. A thorough discussion of crustacean hormones and their known actions is contained in a review by Brown (1952).

Alterations in contents of certain inorganic substances during the premolt and postmolt periods have been studied by Kleinholz and Bourquin (1941b) and by Guyselman (1950). Kleinholz and Bourquin determined the calcium content of exuviae from destalked *Uca* but presented no similar data for the exuviae from normal animals; however, the same investigators determined calcium values for the exuviae from destalked and normal *Palaemonetes* and found no appreciable difference between them. Guyselman found no difference between the calcium contents of exuviae of normal and destalked *Uca pugilator*, although a difference in the total

[1] This investigation is a portion of a dissertation submitted in partial fulfilment of the requirements for the Ph.D. degree in biology at Northwestern University in June, 1952. The author wishes to express his sincere appreciation to Dr. Frank A. Brown, Jr. for his advice and helpful criticism during the course of this investigation.

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ash was observed. The only other investigation pertaining to the metabolism of inorganic substances of *Uca* was that of Edwards (1950) who found that eyestalk removal effected an increase in oxygen consumption. He suggests, without adequate evidence, that the calcium content of the blood is regulated by the sinus gland and that through effecting calcium changes, the sinus gland controls oxygen consumption and other processes known to be regulated by this gland.

The study of growth and molt in *Uca pugilator* has been approached, largely, in a qualitative manner. Abramowitz and Abramowitz (1940) demonstrated that eyestalk ablation leads to substantially increased sizes of the postmolt animals. Kleinholz and Bourquin (1941a) presented data describing weight increases associated with molting in destalked animals, but no dimensional measurements were given; the destalked animals showed a weight increase of about 30%. None of their control animals molted during the experimental period; therefore, there was provided no basis for a comparison of the normal animal with the destalked.

A number of other crustaceans have been investigated with respect to growth, molt, and mineral metabolism, and the mechanisms responsible for their regulation. Baumberger and Olmsted (1928) have discussed osmotic pressure and water changes which are associated with molt in *Pachygrapsus*. Robertson (1937, 1941) has undertaken an analysis of the inorganic composition of British shore crabs; he has studied changes associated with molting, including the absorption of water at ecdysis. Drach (1939) has made excellent studies on several European forms, dealing with the molting cycle in normal animals. Numanoi (1939), Maluf (1940), Smith (1940) and Scudamore (1947) have contributed information on various aspects of the calcium metabolism in crustaceans. Kincaid and Scheer (1952) have discussed the influence of the sinus gland on the tissue composition during the intermolt cycle of *Hemigrapsus nudus*.

Materials and Methods

Animals

The fiddler crab, *Uca pugilator,* was used for these investigations. The animals were collected during low tide in salt marshes at Chappaquoit, Massachusetts, where they occurred in great abundance. Both males and females were utilized, but the great variation in size of the large male chela rendered the males less desirable for this study. All of the animals used were collected during July and August of 1950 and 1951; wet weights and dimensions were taken only on those collected in 1951. Of 300 animals brought into the laboratory, the carapace width ranged from 1.0 to 1.9 cm., with a mean of 1.55 cm. Figure 1 shows the relationship between the cube of the carapace width and the wet weight for female animals. Conspicuous differences in coloration were noted among the animals at the time of collection and in the course of laboratory study; these differences will be discussed later.

The stock animals were kept on sea-water tables in running sea water; the floor of the table was of such a character that the water level varied over its surface from a quarter of an inch to about an inch (sufficient to cover completely an animal). For the purpose of isolating animals in sea water, No. 5 wax-coated vendor cups were punctured on two sides near their bases and arranged on the sea-water table to insure complete water coverage of a contained animal. During other experiments,

including those conducted at Evanston, Illinois, animals were isolated in 4-inch finger bowls containing sea water. The only food available to the animals during the investigations was the planktonic material which was carried in the sea water. No special attempt was made to feed the animals during the course of the work. The temperature of the sea water varied between 21° and 24° C. during the investi-

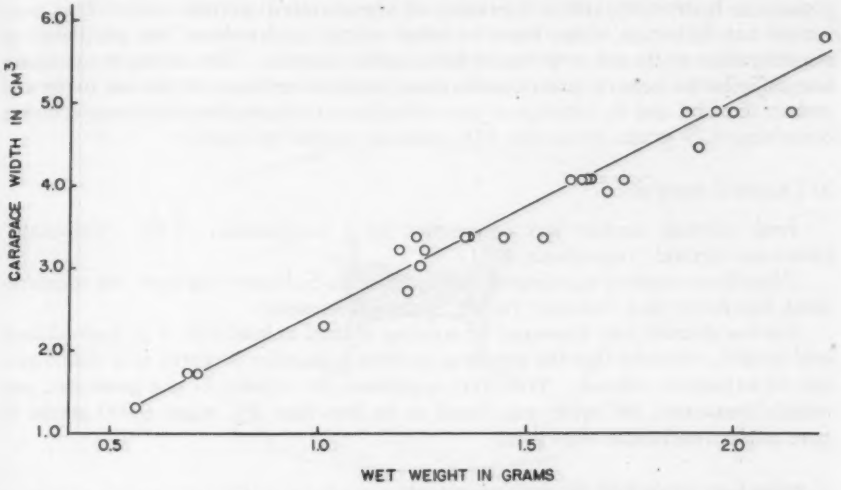

FIGURE 1. The relationship of wet weight to the cube of the carapace width in female *Uca pugilator*.

gations. Records of intensities and durations of illumination were not made; it can be stated, however, that all of the animals in the laboratory were exposed to natural and artificial light for a total period of about 20 hours daily.

Methods

1. Operations

Bilateral eyestalk removal was effected by cutting off both eyestalks at their proximal ends, insuring complete removal of the sinus gland-X organ complexes contained within the stalks.

2. Weights

All weights were determined on an analytical balance.

Wet weights were obtained by leaving an animal for three minutes on a 4-inch filter paper in a dry finger bowl; thereafter, the animal was rolled briefly in a second piece of filter paper, placed in a porcelain crucible (ca. 25 grams) and weighed. To determine whether or not this procedure gave results which were reproducible, a single animal was weighed ten times throughout the course of two hours; the mean weight was found to be 0.9541 grams, with a standard deviation of ± 0.0045 grams.

Dry weights were obtained after an animal had been subjected to one or more dorsal incisions, left in a drying oven at 90° C. for 24 hours, and then left in a desiccator for at least one hour prior to weighing.

Ash weights were ascertained after the crucible containing a dried animal had been left in a Hoskins electric furnace at 1100–1150° C. for ten minutes and then in a desiccator for two hours. All of the desiccators contained colloidal silica gel, potassium hydroxide, and a container of concentrated sulfuric acid. This prevented any hydration of the dried or ashed animal, and reduced the possibility of recombination of the ash with atmospheric carbon dioxide. The ashing temperature was sufficient to convert, quantitatively, pure calcium carbonate to calcium oxide and carbon dioxide, and to decompose pure tricalcium-tertiary-phosphate to a complex containing 0.79 grams of ash per 1.00 gram of reagent phosphate.

3. Chemical analyses

Ionic calcium content was determined by a modification of the "Versenate" titrational method (Guyselman, 1951).

Phosphorus content was determined by the Fisk-Subbarow method; the measurement was made on a Coleman Junior Spectrophotometer.

Carbon dioxide was measured by treating a dried animal with 4 N hydrochloric acid in such a manner that the resulting gaseous expansion occurred in a closed system of adjustable volume. With this apparatus, the volume of gas generated was readily measured; the error was found to be less than 2% when 0.090 grams of pure calcium carbonate were used.

Terminology applied to the molting process

Other investigators have employed various terminologies to describe the various phases and events associated with the molting cycle. The following classification of phases will be used for this work:

1. Intermolt—that period, if any, during which there is a maintenance of a steady-state of the hardened cuticle. It is extremely variable in duration under certain conditions, and may even be lacking in zooecial stages.
2. Premolt—that period during which certain processes lead to epidermal separation.
3. Molt—a time of epidermal separation from the old cuticle, of the formation of a new cuticle, and of the subsequent shedding (ecdysis) of the old cuticle (exuvia).
4. Postmolt—a period of cuticular expansion and hardening, gradually leading either to the intermolt or premolt condition.

Experiments and Results

A. *Premolt*

1. Color changes

It was stated earlier that differences in coloration were observed in freshly collected animals. Since it had been reported that certain changes in pigmentation were a visible indication of active processes leading to ecdysis in some of the

Brachyura (Drach, 1939), the differences in coloration in *U. pugilator* were studied with reference to such a possible relationship.

Cursory observations made during the summer of 1950 on animals retained for some time in the laboratory indicated that the gross pigmentary pattern of an animal in which ecdysis was to occur in two or three days differed from that of individuals ordinarily collected in the field. The former animals were observed to be blue-gray in color, in contrast to the specific patterns of brown, light brown, and purple possessed by the latter and giving the species the colloquial name, "calico backs." However, there seemed to be no indication of any progressive gross color changes preceding the adoption of the blue-gray color which could be regarded as an indication of approaching ecdysis.

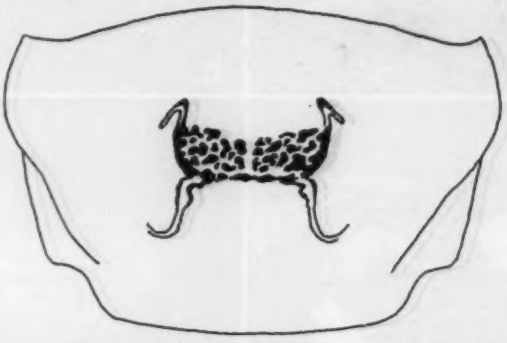

FIGURE 2. Dorsal aspect of the carapace of *Uca pugilator* showing the dorsal crest. The appendages have been removed.

In a group of animals collected on July 29, 1951, the type of coloration in a small region of the carapace served as a means of segregating the animals into three separate groups. Figure 2 is a line drawing of the carapace, showing this distinctive region which will be referred to subsequently as the "dorsal crest." The three groups showed the following differences in the dorsal crest:

Group 1: white patterns on a brown field (Fig. 3a)
Group 2: pale orange and white patterns on a brown field (Fig. 3b)
Group 3: indistinct gray and pale orange patterns on a gray-brown field (Fig. 3c)

During the course of daily observations, a fourth pattern of coloration appeared within animals of group 3. This fourth group had the following characteristics:

Group 4: yellow-orange patterns on a light gray-brown field (Fig. 3d)

Similarly, animals now in group 4 were the source of a fifth pattern which was observed:

Group 5: light yellow patterns on a light blue-gray field (Fig. 3e). Animals in this condition had a gross blue-gray color and were almost identical with those observed in 1950 which were within a few days of ecdysis.

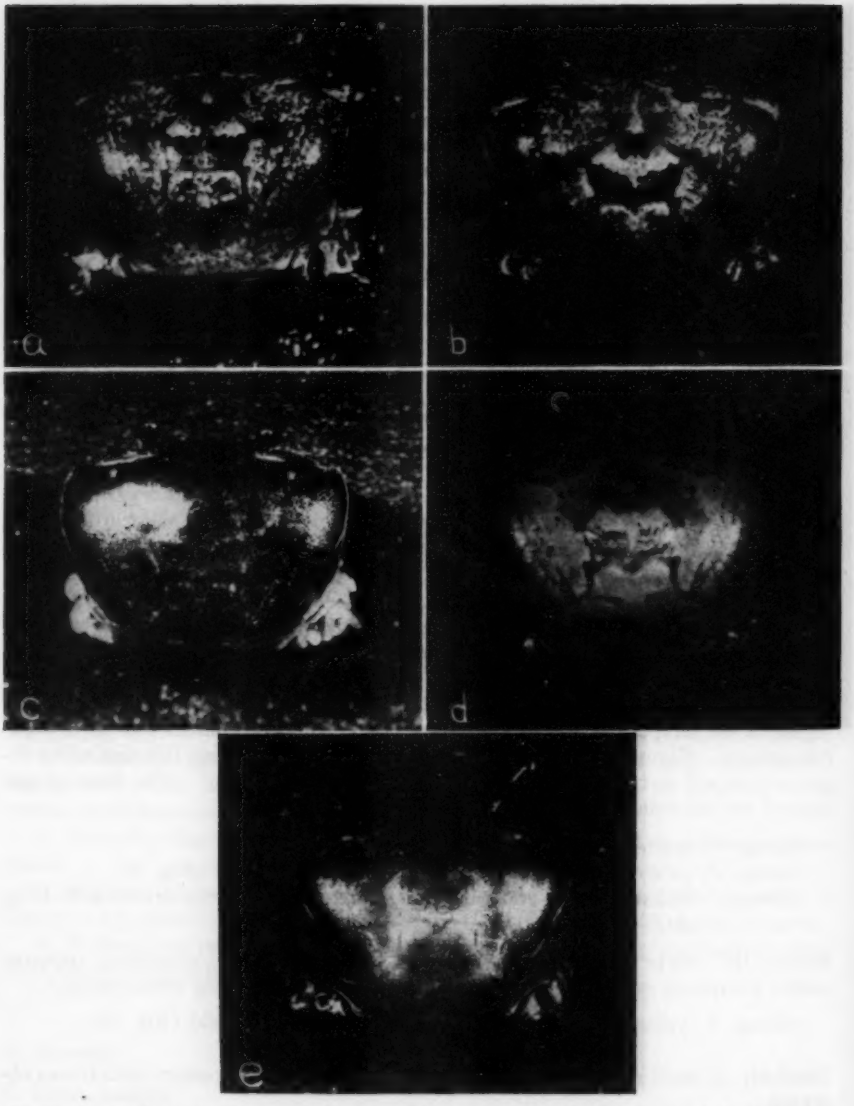

FIGURE 3. Photographs of normal *Uca pugilator* in each of the five stages: (a), stage one; (b), stage two; (c), stage three; (d), stage four; (e), stage five.

Table I shows changes in the number and percentages of animals contained within each of the five groups over a six-day period. It appeared obvious from these data that a progressive change in coloration was occurring in a direction from group 1 to group 5, but evidence was incomplete in that no single animal was seen to make a complete transition from 1 to 5.

Figure 4 shows the changes in group status of a single animal initially in group 1 over the course of 23 days. That this transition occurred in the direction from 1 to 5 was now established beyond doubt. Consequently, the term "stage" was now adopted in place of "group." Furthermore, the length of time that an animal remained in each of stages 2 through 5, under these laboratory conditions, was established. The duration of stage 1 was not determined but the coloration characteristics of an animal immediately following ecdysis are those of a stage 1 animal, as is shown by Figure 5. During a period of a week or two after ecdysis, the maximum time that such animals were observed, no visible changes in coloration occurred. There

TABLE I

Inter-group transition in normal U. pugilator

Date	Percentage of animals in each group					Number of animals
	Group:					
	1	2	3	4	5	
7–29–51	24%	27%	49%	0%	0%	56
7–31–51	24	26	50	0	0	56
8– 1–51	24	25	46	5	0	56
8– 2–51	23	24	24	23	6	52
8– 3–51	15	16	27	26	16	52
8– 4–51	5	18	28	36	13*	50

* Of the seven animals in group five, two underwent ecdysis, three died, and two were sacrificed for inorganic analyses.

is nothing to suggest that any coloration change occurs between ecdysis and the premolt period. Therefore, stage 1 is presumed to extend throughout the intermolt period.

No attempt was made to interpret these changes in coloration at the histological level; however, it can be shown that this transition is correlated with the process of formation of the new cuticle and its separation from the old exoskeleton, and probably also with the partial resorption of the old cuticle. Figure 5a shows a stage 5 animal with a portion of the carapace removed; the new cuticle (with stage 1 characteristics) has separated completely from the old cuticle. Similar examinations were made on animals in other stages; only in 3, 4 and 5 was cuticular separation clearly evident. Considerable variation, however, existed in stage 3 animals with regard to the completeness of cuticular separation; in some cases it was still attached, at least in part, to the carapace while in others it had separated.

During the remainder of the summer and fall of 1951 three additional series of animals were isolated and examined daily for progressive stage changes. The re-

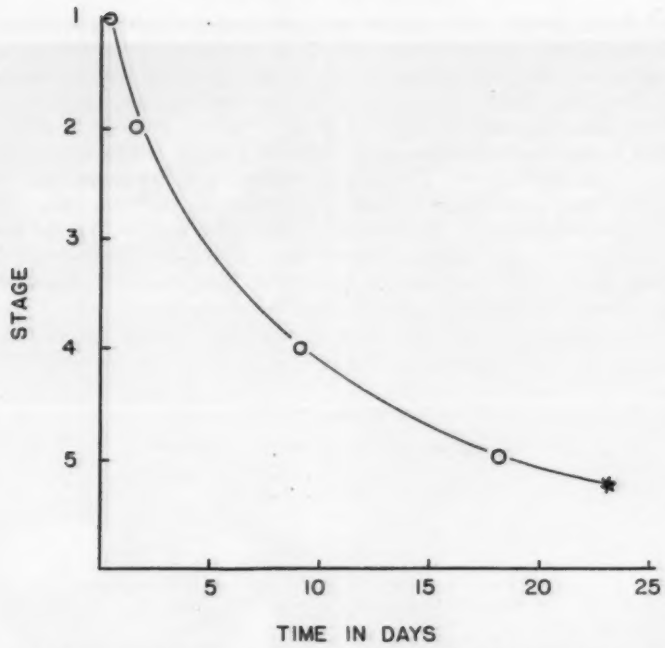

FIGURE 4. Interstage transition for a single, female *Uca pugilator* from stage one until ecdysis; *ecdysis.

sults of these investigations are given in Table II. It appears evident from these results that interstage transition does not occur at the same rate at all times of the year. This is further substantiated by the decreased incidence of ecdysis in series B and C animals.

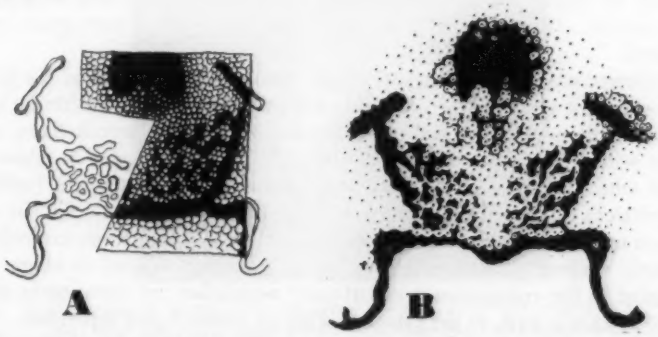

FIGURE 5. Carapace coloration of *Uca pugilator* in stage five (a) showing portion of new cuticle, and carapace coloration in the postmolt condition (b).

During these investigations of the changes in coloration of the carapace in normal animals prior to ecdysis, an attempt was made to determine whether or not a similar series of changes took place in destalked animals. Observations made during the summer of 1950 on destalked animals seemed to indicate that there was little or no difference in gross coloration between animals that had been destalked for a day or two and animals which were within a few hours of ecdysis. Furthermore, it had been shown that one of the effects of bilateral eyestalk removal on *U. pugilator* was a paling of the carapace (Abramowitz and Abramowitz, 1940). Daily observations

TABLE II

Inter-stage transitions at different times of the year during five-day periods. Percentages represent the distribution at the beginning and at the end of five-day periods

Stage	Group A 7-31-51 to 8-4-51		Group B 8-11-51 to 8-15-51		Group C 10-22-51 to 10-27-51	
1	24%	5%	35%	13%	24%	25%
2	26	18	30	27	64	66
3	50	28	27	21	8	6
4	0	36	6	3	0	0
5	0	13	2	0	0	0
Total no. of animals observed	100	64	56	50	30	30
Total no. of animals that molted		2		0		0

on a series of animals destalked July 28, 1951, showed no indication of transitional color changes; in all instances these animals assumed a light gray-tan color and retained to some extent their previous dorsal crest coloration until the time of ecdysis.

2. Inorganic constituents of normal animals

In view of the fact that visible alterations in coloration occurred in normal crabs antecedent to ecdysis in such a regular manner that the approximate time of the subsequent ecdysis could be predicted, a method was provided by which chemical analyses of animals could be made at determinable periods prior to ecdysis. It will be recalled that the staging by coloration had not been perfected in time for the studies made in 1950; therefore, the analyses made at that time could be correlated only with three phases: the first including stage 1 and 2; the second, 3; and the last, 4 and 5. In the analyses to be described in this section, stage 1 animals were restricted to those which, as far as could be determined, had completed their postmolt hardening of the exoskeleton.

Calcium

It has been reported that calcium constitutes 16.3% of the dry weight of a freshly killed *U. pugilator* (Kleinholz and Bourquin, 1941b). This value is based on the average obtained with five animals. Table III contains figures for the dry weight, ash

TABLE III

Inorganic constituents of normal U. pugilator

Animal no.	Sex	Width (cm.)	Stage	Dry weight (gms.)	Ash weight (gms.)	Calcium (gms.)	Calcium as % of dry weight
C99	F	1.6	1	0.7909	0.1627	0.072	9.1
C84	F	1.6	3	0.4903	0.1420	0.058	11.9
B16	F	1.6	3	0.5283	0.1929	0.048	9.0
C42	F	1.65	2	0.6471	0.1703	0.078	12.0
C101	M	1.5	5	0.7754	0.2600	0.084	10.8
C102	M	1.65	1	0.7628	0.3224	0.106	13.8
C103	M	1.7	3	0.8124	0.3048	0.114	14.0
C31	F	1.65	1	0.6010	0.1586	0.072	11.9
D29	M	1.8	2	0.5625	—	0.078	15.4
C111	M	1.0	2	0.1415	—	0.020	14.2
10	M	2.2	1–2	0.7394	0.2704	0.084	11.4
55	M	—	1–2	0.6737	0.2150	0.128	19.0
56	M	—	1–2	0.6483	0.2110	0.101	15.4
57	M	—	1–2	0.8493	0.2662	0.139	16.3
8	M	—	4–5	1.1359	0.4029	0.188	16.5
58	M	—	1–2	0.6518	0.2358	0.096	14.7
59	M	—	1–2	0.6975	0.2290	0.122	17.5
43a	M	—	3	0.7052	0.2382	0.140	19.8
C57	M	—	1–2	0.7569	0.2515	0.124	16.3
6	M	—	4–5	0.8175	0.3058	0.176	20.3

weight, calcium, and calcium as the percentage of dry weight for 20 animals. It will be seen that the amount of calcium ranges from 9.0% to 20.3% of the dry weight, with an average of 14.4% and a standard deviation of ± 3.4%. While 9.0% is the minimal value for an animal in stage 1, a value of 10.8% is found for an animal which appears to be within a day or two of ecdysis. From these data it would seem that there is no correlation between the stage of an animal and the amount of calcium it contains. A value of 9.0% would seem to be the minimal amount occurring in a fully hardened animal.

The most abundant forms of inorganic calcium in decapod crustaceans are carbonate and phosphate. Table IV shows the proportions in which these salts occurred in four completely hardened animals. Calcium carbonate constituted about 27% of the dry weight while calcium phosphate comprised about 9%. The ratio

TABLE IV

Carbonate and phosphate contents of normal U. pugilator

Animal	Sex	Width (cm.)	Stage	Dry weight (gms.)	Calcium carbonate		Calcium phosphate	
					grams	% of dry weight	grams	% of dry weight
C112	F	1.2	3	0.191	0.055	28.8	0.022	11.6
C111	M	1.0	2	0.141	0.033	23.5	0.010	7.2
D29	M	1.8	2	0.562	0.172	30.5	0.049	8.8
D31	F	1.65	1	0.560	0.140	25.0	—	—

of the carbonate to the phosphate in animals under these conditions appears, therefore, to be approximately 3 : 1. Data are not available for all of the five stages in the molting cycle with respect to carbonate and phosphate contents. However, as will be evident later, studies of ashed animals give data which permit one to determine whether any significant alteration in this 3 : 1 ratio has occurred. These later data were obtained from animals representing all five stages.

Ash

At the temperature employed for the ashing of animals, all organic matter was destroyed, calcium carbonate was quantitatively converted to calcium oxide, and calcium phosphate was decomposed to a mixture of its oxides. An average value of 32.7% was obtained for 17 animals in all of the stages; values ranged from 20.6% to 42.2% for animals in stage 1, with a mean of 29.7%. Animals in stages 2 through 5 gave values of from 29.0% to 37.4% with a mean of 33.9% and a standard deviation of ± 2.6%. The greatest variation is found in stage 1 animals, all of which, however, appeared to have completely hardened exoskeletons.

Water

The water content of animals in stages 1 through 5 was found to be 67.9% with a standard deviation of ± 4.7%, values ranging from 57.1% to 74.0% for 13 animals. No significant variation in water content of hard-shelled animals was observed through the five stages. The variation in water content of stage 1 animals during the period of the hardening of the exoskeleton will be treated later. It should be mentioned that the average value of 67.9%, found in these analyses, was obtained with animals that had been kept in running sea water. For a period of ten days, relatively low weights were obtained between 7 P.M. and 8 P.M., while higher weights were found during the morning hours. Figure 6 contains values for the wet weights of an animal during the course of three days prior to ecdysis. It will be seen that during the last two days prior to ecdysis the fluctuations appear arrhythmical, in contrast to a diurnally rhythmic tendency which had been noted during the first seven days of observation. Furthermore, the amplitude of the fluctuations decreases as the animal approaches ecdysis. Immediately after ecdysis the minimal daily weight was obtained at about 6 P.M., while the maximal weight was obtained at about 6 A.M. This aspect of postmolt weight changes will be discussed in a later section. Animals kept in distilled water for 24 hours showed arrhythmic fluctuations and the amplitude of the fluctuation was considerably reduced as compared to the changes in the animal shown in Figure 6. No weight fluctuations of a rhythmic character were observed in animals kept in finger bowls of sea water in the experiments performed in Evanston, Illinois in October and November. The fluctuations observed were, however, essentially of the same amplitude as those reported for animals at the Marine Biological Laboratory during the summer.

3. Inorganic constituents of destalked animals

Calcium

No appreciable difference could be detected between the calcium contents of normal and destalked animals. For a group of animals which had been destalked for

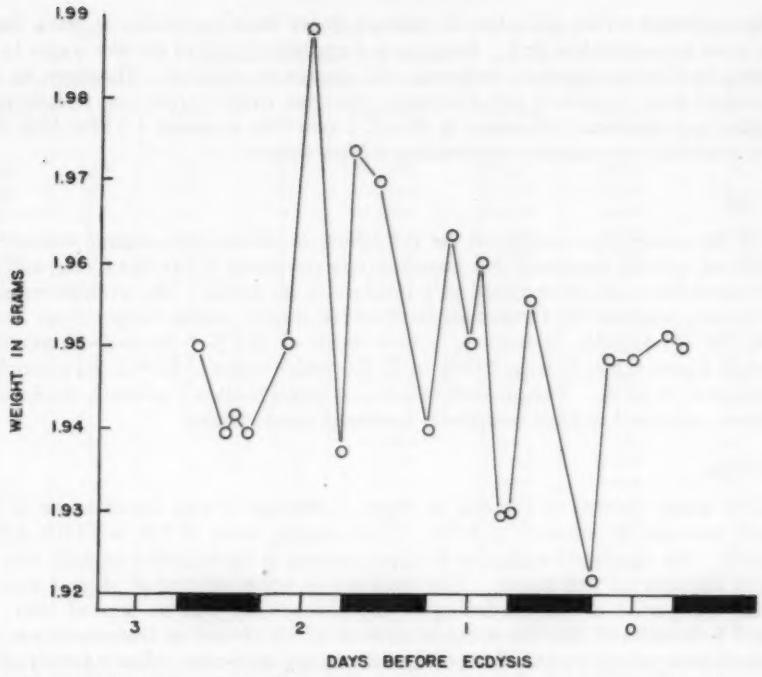

FIGURE 6. Weight changes in single, normal, female *Uca pugilator* before ecdysis. The black bar indicates a period from 6 P.M. to 6 A.M.

three, five, seven, and fourteen days, the mean percentage of calcium was found to be 16.4% with a standard deviation of ± 2.1%, values ranging from 12.9% to 18.7%. These values are well within the range found for normal animals. While calcium is not stored in the hepatopancreas of *U. pugilator* to any appreciable extent (less than 0.5% of the total dry weight in normal animals), one of the effects of bilateral eyestalk removal has been shown to be the complete depletion of calcium from the hepatopancreas within five days after the operation (Guyselman, 1950).

Ash

The ash, expressed as percentage of dry weight, was found to be 36.1% with a standard deviation of ±4.7%, values ranging from 30.6% to 45.5%. Again, the average value is not significantly different from that found for normal animals. That certain changes in the inorganic composition do occur following destalking will be shown by a comparison of the exuviae of destalked animals, cast at various times after destalking, with those of normal animals.

Water

The water contents of destalked animals, prior to ecdysis, can be treated only in an indirect manner. Wet weights were determined for five destalked animals

during periods of six to ten days. The results are given in Table V. It does not appear likely from these data that any significant changes occurred in the water contents during this period. There may be a slight increase in weight for the first few days, but after one week the weights are essentially the same as the initial weights.

Three destalked female animals, weighed at the end of seven, eight, and twelve days after destalking, showed that there was no increase in wet weight when compared to normal animals of the same carapace width. One of these animals, 1.8 cm. in carapace width, weighed 2.06 grams on the seventh day after destalking; another, having the same carapace width, weighed 2.05 grams on the twelfth day after de-

TABLE V

Wet weights (in grams) of destalked U. pugilator

No. of days after destalking	Animal number				
	D4	D5	D6	D7	D18
1	1.682	1.588	1.238	1.848	0.704
2	1.703	1.617	1.242	1.872	0.703
3	1.739	—	—	1.904	—
4	1.726	1.636	1.260	1.921	0.708
5	1.685	—	1.244	1.890	—
6	1.694	—	—	1.878	—
7	—	1.597	1.244	1.898	0.723
8	—	—	1.252	1.924	—
9	—	—	1.242	1.892	—
10	—	—	—	1.884	—

stalking. The third animal, 1.9 cm. in width, weighed 2.64 grams eight days after destalking. It will be seen, by inspection of Figure 1, that these values are well within the range of weight variations that are found in normal animals.

B. *Molt*

One of the processes which characterizes the initial phase of molt is the separation of the epidermis from the cuticle. It will be remembered that this epidermal separation was first noticed in animals which were in stage 3. The period from stage 3 to the termination of molt was from 15 to 20 days in those animals which were examined during the early part of August. The duration of stage 3 appeared to be about 6 days at this time. Therefore, assuming that molting activity of the epidermis commences during the latter part of stage 3, an animal would be engaged for about 10 days in visible aspects of the molting process, a process which is terminated by the abrupt shedding of the old exoskeleton.

Bilateral eyestalk removal, thereby eliminating both sinus gland-X organ complexes, will induce molt in *U. pugilator* (Abramowitz and Abramowitz, 1940). Table VI contains data for a group of animals which were destalked on July 28, 1951; all the animals were staged before destalking. These results, despite the small number of animals used and the variability observed, nevertheless suggest that there is an

inverse correlation between the stage number of an animal at the time of destalking and the time elapsing between destalking and ecdysis. For animals in stages 1 and 2, 18 days elapsed; for stage 3, 14 days elapsed; and for stage 4, 10 days elapsed. It is especially noticeable that stage 3 animals show a dichotomy with respect to the interval between destalking and ecdysis, being of the order of either 6 to 8 days, or 15 to 25 days.

The actual process of shedding the old exoskeleton is relatively rapid; normal animals were observed to complete the casting process in about 15 minutes. Such animals showed little motor activity for several hours prior to ecdysis; occasionally these animals and ones in stage 4 were observed to exhibit non-locomotor move-

TABLE VI

Relationship of color-stage in destalked U. pugilator to interval between destalking and ecdysis

Animal no.	Sex	Carapace width (cm.)	Stage at time of destalking	Number of days after destalking that ecdysis occurred
11	F	1.5	1	15
27	F	1.6	1	18
15	F	1.7	2	19
14	F	1.7	2	21
22	F	1.6	2	17
8	F	1.5	2	14
17	M	1.9	2	14
6	M	1.6	2	27
35	M	1.5	3	18
45	M	1.6	3	14
41	M	1.7	3	27
5	M	1.7	3	15
13	F	1.3	3	8
9	F	1.5	3	6
40	F	1.5	3	12
47	F	1.6	3	14
10	F	1.6	3	18
37	F	1.6	3	18
30	F	1.7	3	7
46	F	1.7	3	13
25	F	1.7	4	15
44	F	1.4	4	5

ments of the walking appendages. This activity was never observed in animals in stages 1, 2, and 3.

The first indication of ecdysis was a separation at the cephalothoracico-abdominal junction. This was followed by a splitting along the lateral margins of the exoskeleton; as the posterior and lateral margins of the carapace split, the animal underwent active muscular movements, gradually withdrawing itself from the exuvia.

Many of the destalked animals did not free themselves completely from the exuviae, certain anterior portions of the alimentary tract remaining attached. This apparent failure of destalked animals to emerge successfully was also observed by Abramowitz and Abramowitz (1940).

1. Uptake of water

Table VII gives the weights of three animals prior to ecdysis, the weights within 15 minutes after emergence, the weights of the cast exuviae, and the increase in weight due to water absorption. In addition, the table includes the weights of the freshly molted animals and the water absorbed, expressed as percentages of the weights immediately prior to ecdysis. It will be seen that the amount of water absorbed at ecdysis is inversely proportional to both weights and carapace widths of the animals before ecdysis. It was found that the product of the cube of the carapace width and the weight of the water absorbed (expressed as the percentage of the weight prior to ecdysis) was relatively constant; for the animals indicated in Table VII, a mean product of 170.0 with a standard deviation of ± 4.7 was obtained. This indicates that the increase in water content at ecdysis appears to be a function of the volume of an animal, with the smaller animals absorbing a higher percentage than the larger ones.

TABLE VII

Changes in wet weights associated with ecdysis in normal, female U. pugilator

Animal no.	Width (cm.)	W1	E1	W2	W3	E1 as % of W1	W2 as % of W1	W3 as % of W1
		(grams)						
1C11	1.7	1.960	0.899	1.740	0.679	45.8	89.0	34.6
51F	1.6	1.619	0.819	1.458	0.658	50.6	91.8	40.6
5F	1.5	1.540	0.868	1.494	0.822	56.4	96.8	53.4

W1 = wet weight before ecdysis
E1 = wet weight of exuvia
W2 = wet weight after ecdysis
W3 = W2 − (W1 − E1) = weight of water absorbed at ecdysis

It was demonstrated earlier that the water content of animals before ecdysis is about 68%. The water content of an animal 15 minutes after ecdysis is approximately 84%, values of 84.5% and 84.1% having been obtained with two animals. Although this value of 84% has been derived from direct determinations using only two animals, it is readily possible to calculate the water contents of the three animals described in Table VII immediately following ecdysis. When this is done, the three values are 82.3%, 89.6%, and 83.1%. The average for these values is 85.0%, which is in close agreement with those obtained by direct determinations.

Sufficient data are lacking for destalked animals with respect to water uptake at ecdysis. However, exploratory research seems to indicate that the increase in weight accompanying ecdysis is approximately the same as that found for normal animals. Kleinholz and Bourquin (1941a) determined the percentage weight increases in destalked *U. pugilator* following ecdysis; however, initial and final weights were not given. They state that of six animals in the 1.6 to 2.0 gram size-class, the average percentage gain in weight after molting is 29.83 ± 7.20%. This value would indicate that, under certain conditions, destalked animals may absorb even less water than normal animals at ecdysis.

2. Weight changes

Figures 7a and 7b show the weight changes in two postmolt animals during a period of five days. It will be seen that at the end of this period the animals have not regained the weight they had prior to ecdysis. None of the animals was weighed at a later time than ten days after ecdysis.

An inspection of the plotted weights in Figures 7a and 7b suggests, again, a rhythmical variation, with the greatest weights occurring at about 6 A.M., and the

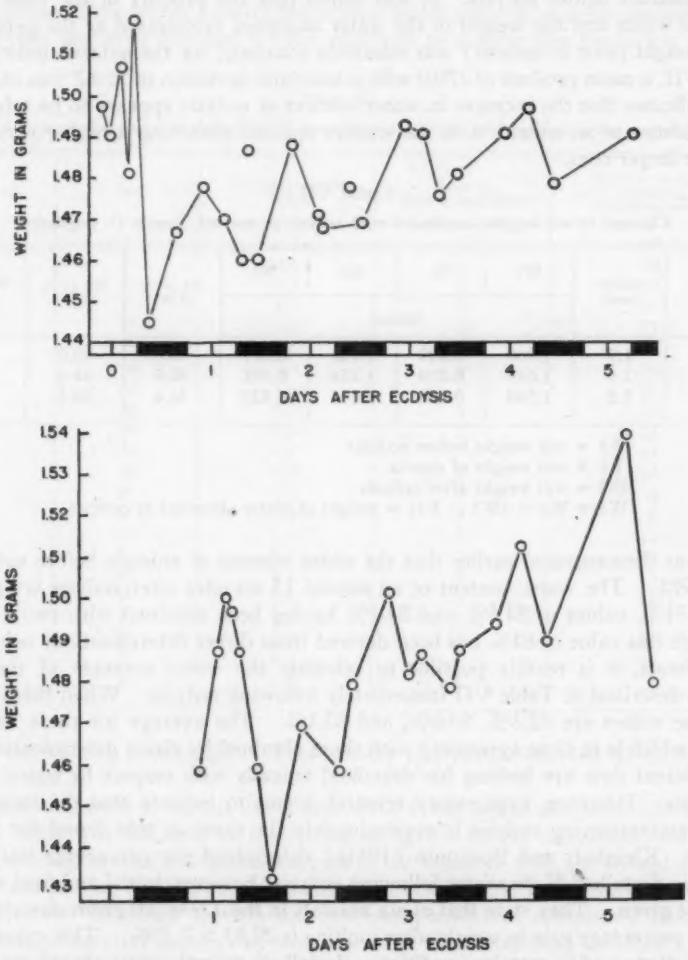

FIGURE 7. Weight changes of two, normal, female *Uca pugilator* (a and b) following ecdysis. The black bar indicates a period from 6 P.M. to 6 A.M.

TABLE VIII

Changes in carapace width in normal and destalked U. pugilator following ecdysis

Animal no.	Condition	Width before ecdysis (cm.)	Days after ecdysis that new dimensions were taken	Width after ecdysis (cm.)	Percentage increase in width
40	Destalked	1.5	0	1.6	6.7
14	Destalked	1.7	2	1.8	5.9
41	Destalked	1.7	3	1.8	5.9
35	Destalked	1.5	5	1.6	6.7
13	Destalked	1.3	7	1.35	3.8
49	Destalked	1.6	8	1.8	12.5
25	Destalked	1.7	8	1.9	11.8
15	Destalked	1.5	18	1.8	20.0
A26	Normal	1.6	1	1.6	0
C105	Normal	1.3	2	1.3	0
51F	Normal	1.6	5	1.6	0
5F	Normal	1.5	5	1.5	0
124	Normal	1.3	7	1.3	0
C11	Normal	1.7	7	1.7	0

smallest at about 6 P.M. The rhythmical consistency of the fluctuations in weights which was obtained for these animals suggests strongly that some factor other than error inherent in the technique in weighing is responsible for this orderly fluctuation.

3. Dimensional changes

Of primary interest is the fact that none of the three normal animals, discussed above in Table VII, showed any change in carapace width during the ten-day period following ecdysis; all three retained their pre-ecdysis dimensions. Three additional animals, the weights of which were not obtained (hence they received relatively little handling), similarly failed to show an increase in width.

TABLE IX

Inorganic constituents of normal and destalked, postmolt U. pugilator

Animal no.	Condition and days postmolt	Dry weight (gms.)	Ash		Calcium	
			(gms.)	% of dry weight	(gms.)	% of dry weight
3E	N 2 hrs.	.1478	.0389	26.4	.010	6.7
C105	N 2 days	.1329	.0395	29.7	—	—
26	N 5 days	.3725	.0960	26.5	.019	5.1
124	N 7 days	.1811	.0564	31.0	.017	9.1
39	D 1 day	.2678	.1042	39.3	.010	3.7
20b	D 4 days	.3692	.1385	37.4	.041	11.0
13	D 7 days	.1474	.0502	35.0	.010	6.8
49	D 7 days	.3015	.1098	36.5	.031	10.2
35b	D 12 days	.2552	.0528	20.7	.013	5.2
31	D 17 days	.3417	.1034	30.2	.021	6.2
15	D 18 days	.3406	.1213	32.5	—	—

Destalked animals, on the contrary, did increase in carapace width and exhibited this increase soon after ecdysis. Table VIII contains values for carapace widths of destalked animals before and after ecdysis, and the percentage increase over the width before ecdysis. For purposes of comparison similar data are presented for the six normal animals. It will be seen, therefore, that at least for a postmolt period of seven days, destalked animals present a striking contrast to normal animals.

4. Hardening

Table IX contains data for the inorganic composition of a group of postmolt animals, including both normal and destalked. One of the most notable differences between the two is the relatively high ash content of destalked forms. It now becomes of interest to examine the composition of the cast exuviae of destalked and

TABLE X

Relationship in destalked animals of the ash and calcium contents of the exuviae to the interval between destalking and ecdysis. Exuviae of two normal animals are included for comparison

Exuvia no.	Days after destalking that animal underwent ecdysis	Dry weight (gms.)	Ash		Calcium	
			(gms.)	% of dry weight	(gms.)	% of dry weight
3E	2 hours	.2430	.1233	50.0	.052	21.5
A18	5 days	.2228	.1409	63.5	—	—
44	6 days	.1919	.0919	48.0	—	—
9	6 days	.2056	.1084	53.0	.058	28.2
A19	7 days	.1570	.0704	44.0	—	—
13	8 days	.1704	.0830	49.0	.047	27.8
30	10 days	.2698	.1296	48.0	.076	28.6
A26	10 days	.2530	—	—	.073	29.0
6	27 days	.3279	.1571	47.0	—	—
41	27 days	.3939	.1919	48.0	—	—
	Normal animals					
55		.4396	.2584	59.0	.122	27.7
5F		.2324	.1444	64.0	—	—

normal animals. This information is given in Table X. Indicated are the dry weights, ash weights, calcium, and the ash and calcium as percentages of the dry weights. It will be seen that animals which undergo ecdysis within five or six days after destalking shed an exuvia which contains about 53% ash; if ecdysis occurs later than six days after destalking, the ash content is about 47%. This value of 47% is found for exuviae of destalked animals which are cast between six and 27 days after destalking. In contrast, normal animals shed an exuvia which contains about 61% ash. The percentage of calcium, however, is about 27% for exuviae from both normal and destalked animals. That the calcium content of exuviae from destalked animals is about 27% was reported by Kleinholz and Bourquin (1941b). It will be seen by an inspection of Tables IX and X that the extent of the reduction in ash content in exuviae of destalked animals, in comparison with normal, corresponds closely with the proportionally higher ash content which is observed in destalked postmolt animals, a value of approximately 10% having been obtained in both cases.

Discussion

One of the effects of bilateral eyestalk removal in *Uca pugilator* has been shown through these investigations to be a reduction of the non-calcium inorganic content of the exoskeleton, and the apparent transfer of this material into the soft tissues of the animal (*cf.*, Tables IX and X). Since the calcium content of the exoskeleton is not appreciably altered by eyestalk ablation, it would appear that the sinus gland-X organ complex exerts a specific influence not on calcium metabolism *per se*, but rather on processes which control anionic metabolism. Such a mode of action would affect the calcium level in an indirect manner. In all probability a similar situation occurred in Koller's (1930) investigations of *Crago;* he reported that the acid-solu-

Table XI

Calculations in terms of various forms of calcium for normal and destalked U. pugilator

Animal no.	Number of days after ecdysis	Condition	Calcium		A as % of ash	B as % of ash
			A calculated as 100% calcium oxide	B calculated as 100% calcium phosphate		
39	1	Destalked	.014	.020	13.4	19.2
20b	4	Destalked	.057	.084	41.0	60.8
13	7	Destalked	.014	.020	28.0	40.0
49	7	Destalked	.043	.063	39.4	57.6
35b	12	Destalked	.018	.026	34.1	49.2
31	17	Destalked	.029	.043	28.0	41.6
3E	0	Normal	.014	.020	36.0	51.2
26	5	Normal	.026	.039	27.0	40.7
124	7	Normal	.024	.035	42.5	62.0
			(values obtained)*		(ash calculated)**	
C112	Not known	Normal	.031	.017	50.0	27.5
C111	Not known	Normal	.018	.008	40.5	17.0
D29	Not known	Normal	.096	.039	53.3	23.0
D31	Not known	Normal	.078	—	42.6	—

* From Table IV.

** Since actual ash weights could not be determined (a limitation of the procedure), they have been calculated by the formula:

$$\frac{\text{ash weight}}{\text{dry weight}} = 0.32$$

ble mineral content of cast exuviae from destalked animals was less than that found in cast exuviae of normal animals. Furthermore, the results of Kleinholz and Bourquin (1941b), indicating no difference between exuviae of normal and destalked *Palaemonetes* with respect to the calcium contents, may be attributable to a similar mode of action.

A consideration of the anionic composition of animals is necessarily limited; quantitative determinations for phosphate and carbonate were made only on normal animals. Nevertheless, it is possible to suggest certain limitations which are imposed by assuming that calcium is bound primarily only to one or the other, or both, of these anions under all conditions. In Table XI calculations are presented for

the phosphate and carbonate contents of a series of normal and destalked animals; these calculations are based on the ionic calcium values which were actually obtained (Tables IV and IX), expressed as oxides of calcium carbonate and calcium phosphate. In each case it was assumed that the calcium is present entirely in the form of these two compounds. In addition, the calculated oxides are expressed as percentages of the ash.

An examination of Table XI indicates that approximately 70% of the ash can be attributed to the oxides of these compounds; about 46% is from carbonate and 23% comes from phosphate. The remaining 30% of the ash probably consists of oxides of silicon, magnesium, aluminum, iron and sulfur since these elements normally occur in crustacean cuticles (Clark and Wheeler, 1922). If the calcium content of *Uca pugilator*, seven days after ecdysis, is assumed to be entirely of calcium carbonate, the oxides which are obtained by incineration will account for 43% of the ash; if the calcium content is assumed to consist completely of calcium phosphate, the resulting oxides would account for 62% of the ash. In contrast to this situation, destalked animals which were analyzed 17 days after ecdysis showed a maximum of 28% ash from carbonate and a maximum of 41% ash from phosphate. From these facts, it would appear quite clear that some other elements were responsible for the remaining ash. If the calcium compound were considered as calcium sulfate, it would account for approximately 70% of the ash. It is known that the sulfur content of lobsters is considerably higher in older animals than in younger animals (Richards, 1951). The possibility exists that the unknown ash component of destalked, postmolt animals contains a high percentage of sulfur; further investigations on the nature of the ash are needed and may be of considerable significance in attempting to explain the role of the sinus gland in mineral metabolism.

That there is an alteration of the calcium: phosphorus ratio in freshly molted animals as compared to premolt animals has been shown by Robertson (1937); his results with *Carcinus maenas* show the integument of normal, freshly molted animals to be considerably higher in phosphorus than in premolt animals. Expressing his values as the ratio of the percentage of calcium to the percentage of phosphorus, it is seen that premolt animals have a ratio of about 9.5 as compared with values of 0.9 shortly after ecdysis, and 1.7 within a week after ecdysis. On the basis of calculations in Table XI, it is not unlikely that a similar situation may occur in *U. pugilator*.

The results of Edwards (1950) with *U. pugnax* are interpreted by that investigator as positive evidence for an increase in weight subsequent to bilateral eyestalk removal. Of a total of ten animals used for one experiment, six showed a weight increase within two days after the operation. However, if the percentage changes in weights of these six animals are expressed as the averages for two, three and seven days after destalking, increases of 3.0%, 3.0% and 5.5%, respectively, are obtained. In contrast, and not discussed by Edwards, similar calculations for animals which showed a decrease in weight after destalking indicate average decreases of 0.5%, 14.4%, 6.3% and 9.3% at the end of one, two, three and eight days, respectively. Yet, on the basis of the data, Edwards concludes that eyestalk removal leads to an increase in weight.

An inspection of Table V might lead one to believe that destalked animals increase in weight. At the end of six, seven, nine and ten days, the percentage increases for five animals are, respectively, 0.7%, 1.6%, 0.3% and 1.9%. However, it should be remembered that the daily fluctuations of wet weights are sufficient to

account for at least a 2% variation which may be considered either as an increase or decrease of the wet weight. While the range of fluctuations in weight of a destalked animal is less than that of a normal animal, it is possible to demonstrate similar variations. Until additional studies conducted over a greater period of time are made, it seems necessary to conclude that eyestalk removal has had little effect on the wet weight of *U. pugilator*, at least during a ten-day period following the removal.

That water metabolism is in some manner influenced by a hormone source in the eyestalk has some experimental verification. Data are inadequate for a thorough consideration of this aspect; nevertheless, they suggest a possible role of the sinus gland controlling the rhythmical fluctuation in weight. Normal postmolt animals appear to exhibit a diurnal fluctuation in wet weight (Figs. 7a and 7b). Normal premolt animals also show diurnal fluctuations, but as ecdysis is approached, the fluctuations appear to become arrhythmic. Although destalked premolt animals show minor fluctuations, they were not of a rhythmical character. It will be recalled that these rhythmical fluctuations in wet weight were observed only in animals which were kept in running sea water at the Marine Biological Laboratory. Such a rhythm was not observed in animals which were kept in finger bowls with either sea water or distilled water; under these conditions the sea water was subject to evaporation, while neither medium was aerated to the extent of normally running sea water. These differences may well account for the observed absence of a rhythm under these conditions.

An hypothesis of a rhythm in water-uptake, controlled by the sinus gland-X organ complex and with the phase of wet-weight change as described in this report, would suggest a daily fluctuation in blood calcium concentration of normal animals. If such were the case, it would support an assumption made by Edwards (1950), although the mechanism of control would not be that suggested by Edwards. Finally, it would explain why a more pronounced rhythm is seen in normal postmolt animals than in animals immediately prior to ecdysis, since it is generally thought that the titer of sinus gland hormone is lower just before ecdysis than after ecdysis.

It was observed that normal animals showed no increase in carapace width during a ten-day period following ecdysis, in contrast to the situation in destalked forms (Table VIII). The apparent failure of normal animals to assume greater dimensions during the immediate postmolt period has a number of possible explanations:

1. The environmental factors which prevail in the normal molting habitat may be significantly different from those existing in the laboratory, and may be favorable for a growth response. Normally, animals in the field presumably have an adequate food supply at their disposal; such may not have been the case in the laboratory, since the only food available to the animals was that which was brought in by the running sea water. Changes in the chemical composition of sea water in the salt marshes, effected by drainage, may well exist and be responsible for differences from the conditions in the sea water brought into the laboratory. Following summer rains, a hypotonic condition might have an effect on the amount of water which is absorbed by an animal at ecdysis. The sea water which is pumped into the laboratories at the Marine Biological Laboratory comes from an area which is not subject to any appreciable variation.

2. Since the yearly cycle for *U. pugilator* has not been observed, a situation may exist which is similar to that of certain crayfish. Scudamore (1947) found that

Cambarus underwent two yearly molts, growth occurring to a much greater extent in the late summer molt than in the spring molt. Inasmuch as the frequency of molting in normal animals was extremely low during the July-August period, there may well be normally an early summer molt at which time animals exhibit substantial increases in carapace width.

3. Another possibility, and one which is realized in some crustaceans, is that a dimensional increase following ecdysis may be considerably reduced or even lacking under certain conditions. Lloyd and Yonge (1947) found that the growth rate of *Crangon vulgaris* declines as the animals approach maturity and that females which carried eggs showed no increase in length after ecdysis. Hoglund (1943) reported that molt without growth occurs in *Leander squilla* during the winter. Inasmuch as the normal animals on which measurements were taken during the present investigations probably represent adult individuals, it is possible that they had already attained their maximum dimensions. If such an hypothesis is correct, then any additional molts which might occur after such maximal dimensions have been established may serve only to facilitate the regeneration of lost appendages or the repair of damaged portions of the cuticle.

Summary

1. A transitional series of color changes which occurs during the premolt period of normal animals is described. These changes have been arbitrarily assigned to five stages and the duration of each stage measured. The last four of these are associated with hypodermal activity anticipating ecdysis.

2. The inorganic constituents of normal and destalked animals are compared for the premolt and postmolt condition.

3. Certain physical aspects of ecdysis are treated in a quantitative manner. These include the absorption of water and dimensional changes.

4. The results suggest that the sinus gland-X organ complex plays a role in the regulation of water metabolism and in the metabolism of inorganic constituents.

5. Evidence is presented for a diurnal rhythm of water-uptake under the control of the sinus gland-X organ complex.

6. The possibility that molt and dimensional increase are separate factors under certain conditions is discussed.

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RESPIRATION AND IODINE UPTAKE IN ASCOPHYLLUM

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The brown algae are noted for their high iodine content. They have the property of removing iodine from sea water and accumulating it in their cells in concentrations many times that of sea water. This phenomenon of accumulation against a concentration gradient has been observed for several elements in a variety of tissues. There is considerable evidence that aerobic metabolism is necessary for accumulation (Hoagland, 1940), involving the cytochrome system (Robertson and Turner, 1945; Hoagland and Broyer, 1942), and, perhaps, the Krebs cycle (Machlis, 1944).

Since the mechanism of iodine uptake by the algae might well parallel the uptake of salts previously investigated, it is of interest to know whether iodine uptake is related to respiration. To determine this, three types of experiments were carried out:

1. General nature of respiration by the use of oxidizable substrates and inhibitors.
2. Effect of these substrates and inhibitors on iodine uptake.
3. Iodine uptake in nitrogen.

Materials and Methods

The brown alga, *Ascophyllum nodosum* (Linn.) LeJolis was selected as a suitable species for respiration and iodine uptake measurements because (1) it accumulates iodine; (2) reproducible samples could be obtained from day to day by cutting segments; (3) it remains vegetative in the locality used throughout the summer season; (4) it is readily available.

Respiratory measurements. The alga was gathered in the late afternoon, sliced transversely into segments 0.25 mm. thick with a razor blade, and washed overnight in darkness in a running sea water aquarium at a temperature of 18 to 22° C. Segments of this thickness had a respiratory rate of 0.72 μl. O_2 per hour per mg. dry weight (103 μl. O_2 per hour per mg. nitrogen), a rate considerably higher than segments one and ten mm. in thickness (Table I). This suggests that penetration of oxygen into the tissue is an important factor in determining the absolute respiratory rate of this alga. The segments were cut from the distal region between the first and second air bladders; the respiratory rate of segments from this region was lower than that of segments from younger tissue and higher than that of segments from older tissue (Table II). This is evidence that a respiratory gradient exists in this species. Fifty to 400 segments were introduced into each flask, the number being constant in any one experiment. When the latter number was employed,

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TABLE I

Respiratory rate of segments of various thicknesses. Each figure is the average of 2 to 4 determinations. Tissue starved

Thickness in mm.	No. segments in flask	µl. O_2 per hour		
		Per no. segments	Per mg. D.W.	Per mg. N
0.25	400	84.4	0.72	103
1.00	100	48.3	0.57	82
10.0	10	49.4	0.38	72

the number was determined volumetrically. The respiratory rate was proportional to the number of segments used, indicating that oxygen in the flask was not limiting.

Measurements of oxygen uptake were made in darkness in standard Warburg flasks of approximately 15 ml. volume, maintained in a water bath at 25° C. and shaken at 80 r.p.m. Manometer readings were made every half hour after an equilibration period of 30 minutes. Oxygen uptake was measured as the amount of oxygen taken up by the segments during the entire experimental period of three hours. In the preliminary experiments comparing segments of various thickness and from tissue of different ages, the calculations were based also on dry weight and total nitrogen content of the segments. In general, the basal medium consisted of van't Hoff sea water minus calcium.

The effect of the following substances on respiration was measured: sucrose, mannose, succinic acid, malic acid, sodium pyruvate, malonic acid, sodium azide, iodoacetic acid, sodium fluoride, and potassium cyanide. The experiments with sucrose, mannose, azide, iodoacetic acid and cyanide were carried out at pH 6.8, with segments in van't Hoff sea water solution minus calcium as controls. The experiments with succinic, malic, pyruvic and malonic acids and fluoride were carried out in phosphate-citrate buffers of pH 4.5, 5.5 and 6.5, with segments in buffers of pH range 4.5 to 6.5 as controls. The buffers were made up, and the substances tested dissolved, in van't Hoff sea water solution minus calcium. The respiratory rate was 15 per cent less in van't Hoff sea water than in natural sea water; at pH 6.5 (phosphate-citrate buffer) the rate was decreased an additional 15 per cent.

TABLE II

Respiratory rate of segments of different ages. Each figure is the average of 2 to 3 determinations

Age of tissue	µl. O_2 per hour			
	Starved		Not Starved	
	Per mg. D.W.	Per mg. N	Per mg. D.W.	Per mg. N
Youngest	1.07	200	1.52	659
Intermediate	0.60	109	1.19	361
Old	0.23	69	0.71	380

TABLE III

Effect of sucrose and glucose on respiration. Each figure is the average of 2 to 4 determinations. Tissue starved

Concentration in *M*	Per cent change over control	
	Sucrose	Glucose
0.02	8	0
0.05	20	0
0.10	− 2	0
0.20	− 7	15

Iodine uptake measurements. Segments for iodine uptake measurements differed from those used in respiration studies in being one cm. in thickness. Despite the difference in respiratory rate (Table I), the departure was made to obtain a more uniform counting geometry of iodine taken up than was possible with the thinly sliced segments. They were, however, similarly gathered and washed. The segments were placed in 25 ml. rubber-stoppered Erlenmeyer flasks (10 to a flask) containing 3 ml. of van't Hoff sea water solution to which had been added potassium iodide to give a concentration of 0.05 p.p.m. iodide (the accepted concentration in natural sea water). Iodine^{131}, supplied in $NaHSO_3$, was added to the solution in amounts giving approximately 14 μc. per experimental flask. The flasks were rotated slowly on a turntable.

The iodine^{131} removed by the segments during the experimental period (3 to 6 hours) was determined by obtaining counts of the radioactivity present in the seg-

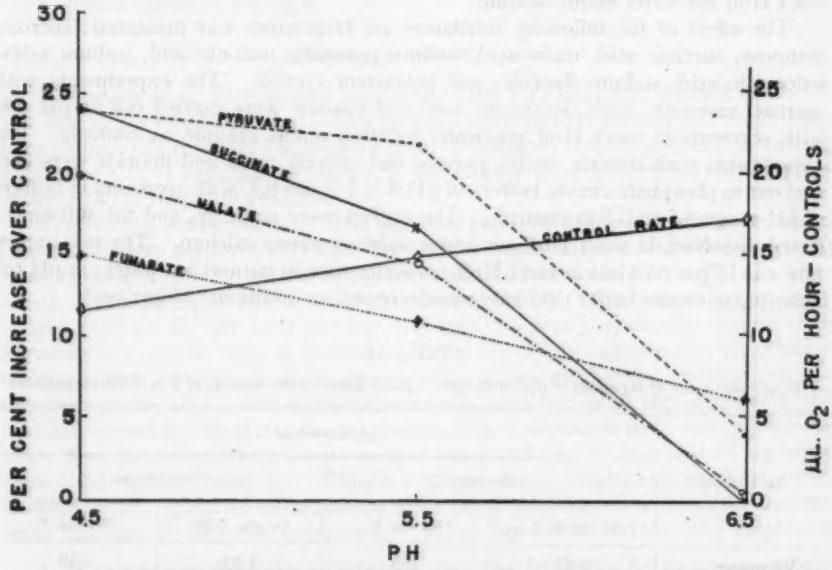

FIGURE 1. Oxygen uptake by Ascophyllum segments in the presence of respiratory intermediates, 0.025 *M* (except pyruvate, 0.0025 *M*.). Segments in phosphate-citrate buffered van't Hoff sea water minus calcium.

ments and in the solutions before and after the segments were exposed to them. Before the segment counts were made, the segments were removed from the solutions and washed by six successive rinses with a non-radioactive solution, otherwise similar to that in the flasks. Each rinse contained 15 to 20 ml. of solution. By the sixth rinse no further removal of radioactivity from the segments took place. The excess rinse solution was blotted from the segments with a paper towel, and the segments transferred to a counting pan. They were evenly spaced so that a constant geometry

TABLE IV

Effect of various concentrations of respiratory inhibitors on oxygen uptake and on uptake of iodine^{131}

Inhibitor and concentration (M)	Per cent inhibition	
	O_2 uptake	I^{131} uptake
Potassium malonate		
pH 4.5		
0.005	16	—
0.010	47	—
0.025	87	79
0.050	81	—
Sodium azide		
pH 6.5		
0.0001	8	77
0.001	84	94
0.01	87	96
pH 6.5		
0.0001	—	71
0.001	13	95
0.01	75	96
Potassium cyanide		
pH 7.5		
0.00001	42	—
0.0001	66	—
0.001	71	—
0.005	58	42
0.05	61	84
Potassium fluoride		
pH 4.5		
0.0025	29	—
0.005	52	—
0.025	81	—

was established for each set of segments counted. After counting, they were returned to the radioactive solutions for the next time interval.

The radioactivity of the solutions was determined by counting two-ml. aliquots of the solutions before and after the segments were exposed to them.

All counting rates were determined by taking at least 2×10^5 total counts, so that the statistical error is below 2 per cent. The counting rates thus determined were then corrected for background and decay of the iodine^{131}.

When a nitrogen atmosphere was desired, the flasks containing the segments were kept in darkness in a vacuum desiccator filled with nitrogen. Before nitrogen was

admitted the desiccator was evacuated and the gas passed through alkaline pyrogallol.

The effect of the following substances on iodine uptake was measured: sucrose, glucose, iodoacetic acid, potassium cyanide, and sodium azide. These substances were dissolved in van't Hoff sea water solution, pH 6.8, minus calcium, to which the isotope was added.

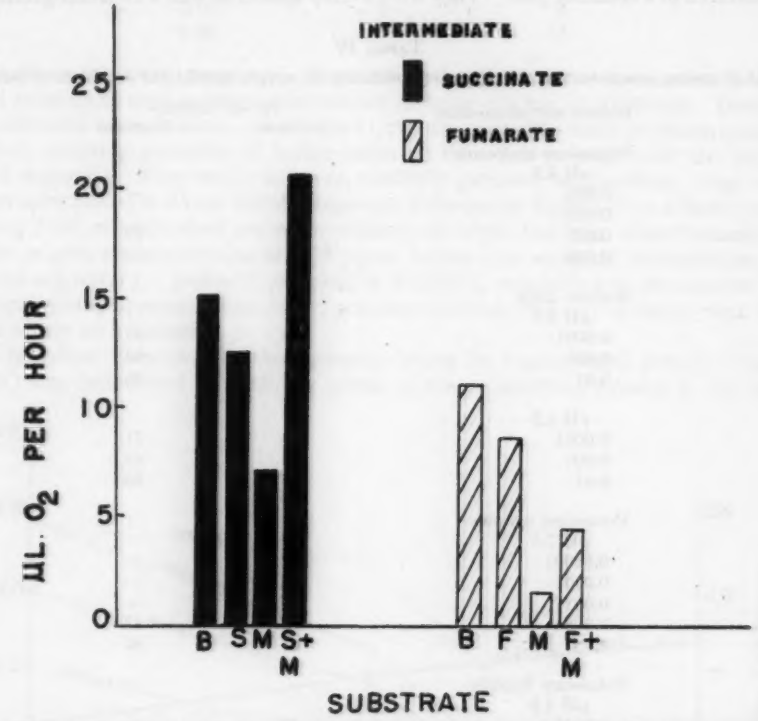

FIGURE 2. Reversal of malonate inhibition of oxygen uptake by Ascophyllum segments. B = basal medium (van't Hoff sea water minus calcium, buffered with phosphate-citrate at pH 4.5); S = succinate; M = malonate; F = fumarate. Concentration of intermediates, 0.025 *M*. Malonate concentration in succinate reversal, 0.01 *M*; in fumarate reversal, 0.05 *M*.

RESULTS AND DISCUSSION

The respiratory quotient of the Ascophyllum segments was 0.9. Since the enzyme systems involved in Ascophyllum respiration are not known, it was thought feasible to make a survey of the effect of substances that are known to serve as respiratory substrates and inhibitors in other types of cells. Of the sugars tested, sucrose and glucose stimulated oxygen uptake of the segments up to 20 per cent, as indicated in Table III. Mannitol, a storage product of Ascophyllum, had no effect

in concentrations of 0.01 and 0.001 *M* and was inhibitory at 0.1 *M*. When the acids, succinic and malic, were tested for their effect on oxygen uptake in van't Hoff sea water at neutral or alkaline pH they stimulated only in high concentrations or after prolonged exposure. When tested at pH 4.5, however, these acids were stimulatory in the concentrations and to the extent given in Figure 1. The respiratory rate of the control at the low pH values was less than at pH 6.5 (Fig. 1). The fact that these acids were effective only at the low pH is in accord with the theory that they enter the cell chiefly in the undissociated state (Baron, 1950; Simon and Beevers, 1951).

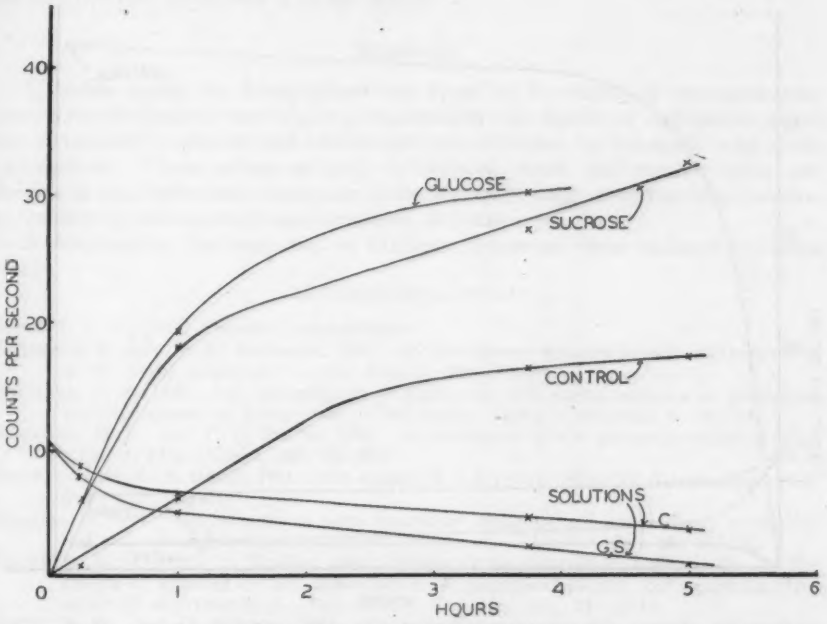

FIGURE 3. Uptake and removal from solution of radioactive iodine by Ascophyllum segments in the presence of added sugars, 0.025 *M*. Segments in van't Hoff sea water minus calcium, containing 0.05 p.p.m. iodide.

The data showing that these acids, known to be intermediates in metabolism of various animal and plant cells, can be oxidized by Ascophyllum segments are suggestive of the presence of a 4-C acid system in this alga.

Additional evidence that this system is present was found in the action of substances inhibitory to respiration of other types of cells: potassium cyanide, sodium azide and iodoacetic acid inhibited oxygen uptake (Table IV). Malonic acid and sodium fluoride inhibited when the pH was lowered to 4.5 but did not inhibit at pH 6.5 (Table IV), again compatible with the theory that these acids enter in the undissociated state. Attempts to reverse the inhibition caused by malonic acid by the

addition of succinic and fumaric acids were successful only when the intermediates were present from the start along with the inhibitor (Fig. 2).

These results suggest that heavy metal enzymes, dehydrogenases, and glycolases are functioning in Ascophyllum segments. Anaerobically the segments utilized malic acid, glucose and pyruvic acid, in the order given, while respiring in nitrogen when carbon dioxide production was followed from a bicarbonate buffer.

The amount of iodine^{131} taken up from van't Hoff sea water by the segments increases up to the first hour of exposure to the element; beyond that time no further increase in radioactivity takes place. Since the segments already had iodine in

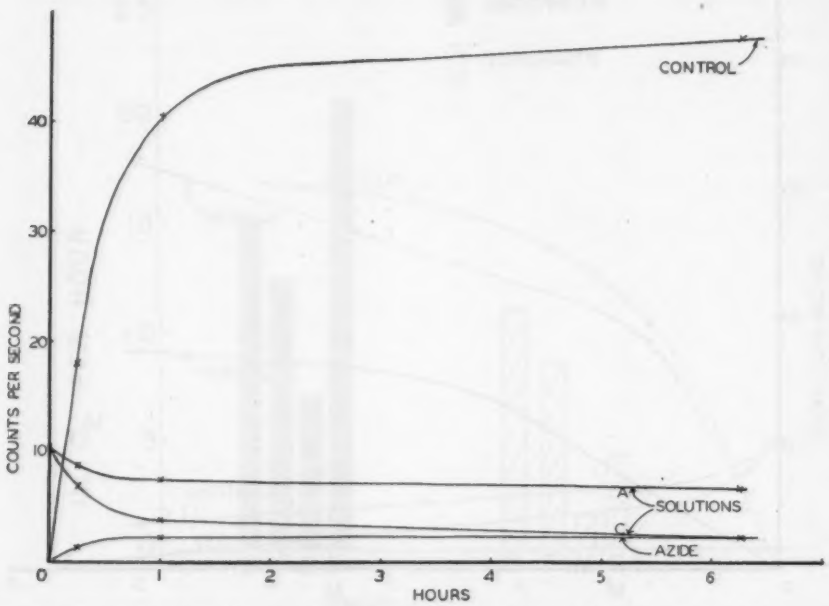

FIGURE 4. Uptake and removal from solution of radioactive iodine by Ascophyllum segments in the presence of sodium azide, 0.001 *M*. Segments in van't Hoff sea water minus calcium, containing 0.05 p.p.m. iodide.

them, having lived in natural sea water until the experimental period, the removal of radioactive iodine has been interpreted as meaning that iodine in the solution is being exchanged for iodine in the segments (Kelly and Baily, 1951). When the ratio of iodine^{131} to iodine^{128} inside the segments becomes equal to the same ratio outside, then net changes in radioactivity cease, indicating an equilibrium has been reached.

When glucose and sucrose were added to the solution in a concentration of 0.025 *M* the uptake of iodine was stimulated, as measured by the increase in radioactive iodine in the segments and its decrease in the solutions. This stimulation was apparent more or less immediately (Fig. 3). Potassium cyanide, iodoacetic acid and sodium azide inhibited iodine uptake (Fig. 4). A comparison of the in-

hibitory concentrations and amount of inhibition of both iodine uptake and respiration (Table IV) shows that iodine uptake is more completely inhibited than respiration by comparable concentrations. This suggests that only a fraction of aerobic metabolism is involved in iodine uptake, an interpretation analogous to that given by Commoner and Thimann (1941) to their experiments in which iodoacetic acid completely inhibited growth at concentrations only partially inhibitory to respiration.

The inhibition by cyanide and azide suggests that iodine uptake is an aerobic process. This interpretation is further strengthened by the fact that segments maintained in a nitrogen atmosphere during the period of iodine uptake show a 50 to 75 per cent decrease in amount of iodine uptake.

Summary

1. Iodine uptake by Ascophyllum was found to be related to the respiratory process on the basis of the following experiments: the uptake of radioactive iodine was stimulated by glucose and sucrose and was inhibited by iodoacetic acid, azide and cyanide. These substances and, in addition, malic and succinic acids and fluoride in turn influenced respiration of the segments, suggesting that they function as respiratory intermediates and inhibitors in the species.

2. Maintaining the segments in nitrogen decreased their radioactive iodine uptake.

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REPRODUCTIVE CYCLE IN CYPRINA ISLANDICA

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Cyprina (Arctica) *islandica* is a clam closely resembling in general appearance the more common species, *Venus mercenaria*. Externally it differs from the latter by having a shaggy, black periostracum which covers the shell. Formerly it was considered to be exclusively a European species but later it was found to be widely distributed on both sides of the North Atlantic. In Europe it ranges from the coasts of England and Norway to the Arctic, while on our Atlantic Coast it is found as far south as Cape Hatteras. However, since it is a cold water species, it is not very abundant south of New England.

A review of the literature shows that many aspects of the biology of *Cyprina islandica* have never been studied. According to Jørgensen (1946) practically nothing is known about the reproduction of this species and, therefore, virtually no literature exists on the subject. Lack of knowledge of the biology of *C. islandica* of our Atlantic Coast is probably due to the difficulty of collecting these clams because they live comparatively far from shore and in deep water. Moreover, since until recently they were not caught commercially, the specimens could not be readily obtained from fishermen.

The situation was changed in 1943 when, in an effort to increase war-time production of sea food, an extensive fishery of *C. islandica* was developed in the waters of Rhode Island and Massachusetts. Since then, samples of these clams have been more easily obtainable. As the fishery expanded and the clams began to appear as a canned product, and in fresh condition, the U. S. Food and Drug Administration in 1944 officially accepted "ocean quahog" as the common name for *C. islandica* (Rhode Island Report, 1945). In this article, for the sake of brevity, we shall call them clams.

Since the beginning of the war-time intensive fishery, new beds of the clams have been discovered. As a rule, these beds are located at a depth ranging from 70 to 120 feet, and are usually confined to either sand and mud or sticky mud (Arcisz and Sandholzer, 1947). Samples of the clams for our studies were collected from such beds located off Point Judith, Rhode Island in approximately 100–120 feet of water. They were collected often enough to record any significant changes in the conditions of the gonads. The clams constituting the samples were adults, several years old, and averaged 3½ to 4 inches in length.

The lack of instruments prevented us from recording the temperature of the water directly over the beds at the time the samples were collected. Fortunately, information on the seasonal temperature changes in that general area is available from the data of Merriman and Warfel (1948), who recorded the bottom temperatures at monthly intervals between September, 1943 and May, 1946 in the area near Point Judith. Because of the proximity of the localities where these temperatures were recorded to our clam beds and because of the strong tidal movements in that

general area, it is thought that there should be no pronounced local temperature differences.

Merriman and Warfel (1948) also gave data for bottom salinity of the same general region for the same period. Generally, the salinity was quite steadily maintained between 31.0 and 32.8 p.p.t., suggesting that this is an optimum salinity range for the existence and propagation of *C. islandica*.

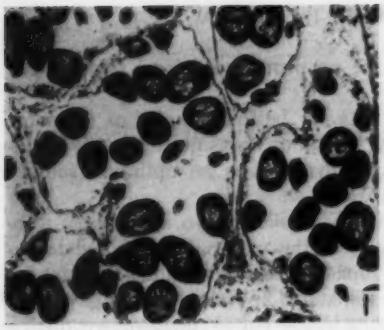
In studying the seasonal gonadal changes of *C. islandica* two approaches were used. The first consisted in histological studies of the material, which led to accurate determination of the condition of the gonads of each individual. Later on this information was used for the second, the statistical, approach, which gave us a quantitative conception of the successive changes in the gonad development during the year and of the progress of spawning during the season. The histological material will be discussed first, while the quantitative data and their analyses will be presented later and summarized in Tables I and II and Figure 13. Figure 13 was made the last figure of the article because of the convenience of not disturbing the continuity of the photomicrographs constituting Figures 1–12 inclusive.

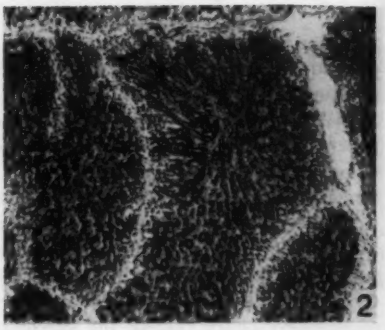
We may begin by considering the condition of the gonads of *C. islandica* just before the beginning of spawning. During the period of our studies this condition was reached by the end of June or early in July, when the temperature was near 13.0° C. Ripe female gonads were characterized at that time by extended follicles containing almost exclusively large, ripe ovocytes (Fig. 1). The walls of the follicles were touching each other, thus leaving virtually no space for the vesicular connective tissue. In males ripe spermatozoa predominated, occupying the largest portion of the follicular spaces, while cells of early stages of spermatogenesis were few in number and were confined to the area near the follicular walls (Fig. 2).

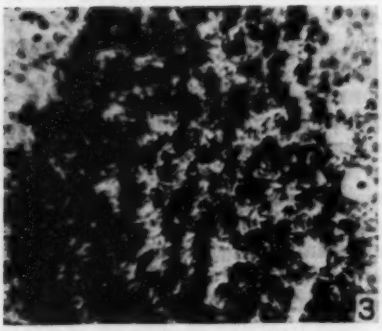
Even a superficial study of the gonads of ripe males often showed masses of "packed" spermatozoa, a condition caused by the arrangement of spermatozoa in the follicles in such a way that their heads were "packed" together creating the impression of a dense band (Fig. 3). A similar phenomenon was observed in *V. mercenaria* (Loosanoff, 1937a, 1937b) and several other lamellibranchs.

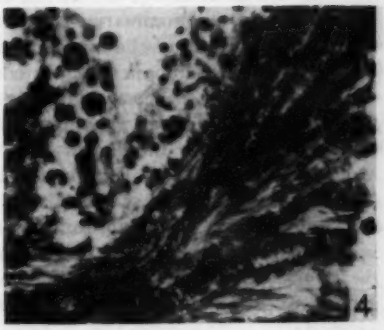
In addition to normal sex cells the follicles of many males also contained numerous multi-celled spherical dark bodies which were usually closer to the periphery of the follicles than to their centers (Fig. 4). Detailed examination of these bodies showed that they were cells of atypical spermatogenesis, noticed by Loosanoff (1937a) in *Venus mercenaria* and described by Coe and Turner (1938) in *Mya arenaria*. These cells apparently develop from the same type of spermatogonia as the normal ones but behave in a strikingly different way, undergoing several nuclear divisions, while still surrounded by the original mass of cytoplasm contained within the same cell membrane. Such abnormal bodies may contain from two to 16 nuclei (Fig. 5). It appears, as was noticed by Coe and Turner (1938), that in some cases such groups of nuclei may break apart and continue further individual development finally reaching the stage of spermatozoa. More often, however, they become pycnotic and are quickly cytolyzed.

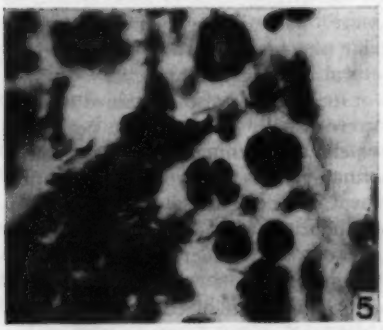
As is usual with lamellibranchs (Nelson, 1928a; Loosanoff, 1937b; Loosanoff, 1942), the entire population of *C. islandica* does not reach ripeness at the same time. We estimate that in the case of these clams only approximately 75 per cent were











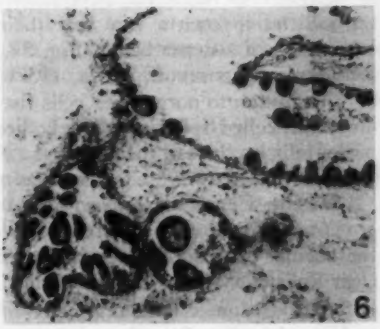


FIGURE 1. Gonad of ripe female. × 112.

FIGURE 2. Gonad of ripe male containing masses of spermatozoa ready to be discharged. × 112.

FIGURE 3. Section of male gonad showing "packed" spermatozoa. × 475.

FIGURE 4. Cells of atypical spermatogenesis occupying the periphery of a follicle containing ripe spermatozoa. × 475.

FIGURE 5. Different groups of cells (spherical) of atypical spermatogenesis. Normal spermatozoa are also present. × 1100.

FIGURE 6. Gonad of female in advanced stages of spawning. × 112.

TABLE I

Frequencies of occurrence of clams in different stages of sexual development at different dates between March 22 and November 1. Stage A: some ripe cells but generally unripe; Stage B: ripe but had not begun to spawn; Stage C: partly spawned; Stage D: completely spawned

Stages	Dates															
	3–22	4–5	4–19	5–3	5–17	5–31	6–14	6–28	7–12	7–26	8–9	8–23	9–6	9–20	10–4	10–18	11–1
A	10	7	8		3	4		5		0	0	0	0	0	0	0	0
B	3	2	2		1	3		13		5	4	8	6	1	0	0	0
C	0	0	0		0	0		2		3	4	10	12	6	3	8	1
D	0	0	0		0	0		0		0	0	1	3	6	4	9	5
Total	13	9	10		4	7		20		8	8	19	21	13	7	17	6

ripe or nearly ripe at the beginning of the spawning season. In the others the gonads still contained a large number of unripe cells. These conditions were especially noticeable in retarded males, the gonads of which contained predominantly the cells of early stages of spermatogenesis, although a few sperm were already present in the centers of some of the follicles.

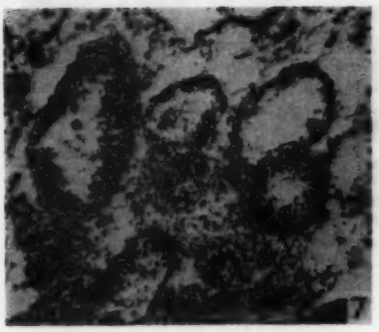
Judging by the condition of the gonads of both sexes, spawning began late in June or early in July when the water temperature over the beds was about 13.5° C. This temperature closely approached that reported by Nelson (1928b) for two other deep-water lamellibranchs, Astarte and Venericardium, which spawn near 12.0° C. Towards the end of July between 30 and 40 per cent of the clams were partly spawned but, as a rule, the quantity of spawn discharged remained small even then, thus indicating that a heavier and more general spawning was yet to follow. This was true because during August spawning continued, involving larger groups of the clam population, and toward the end of this month individuals of both sexes in advanced spawning conditions were becoming more common. In the females constituting this group the gonadal follicles were either empty or contained a few undischarged eggs (Fig. 6). In the males, most of the content of the follicles was discharged but the abnormal cells were still retained in large numbers, being confined to the periphery of the follicles where they were found among the normal cells

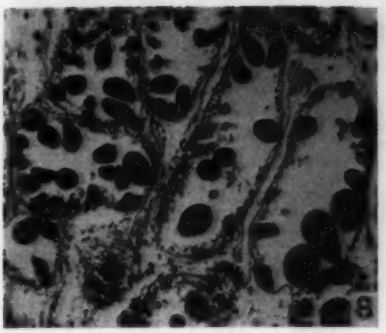
TABLE II

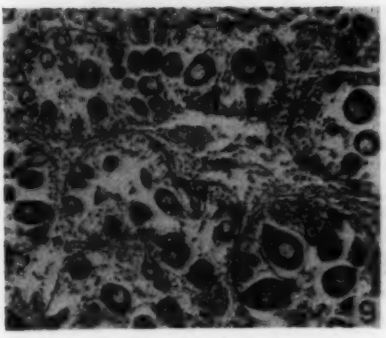
Cumulative percentage of clams at different stages of sexual development between March 22 and November 1. Stages as defined in Table I

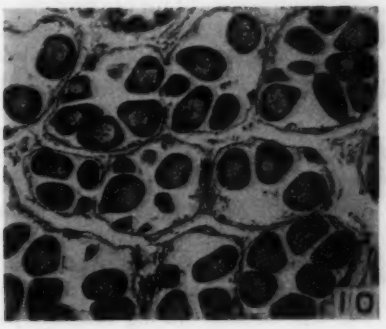
Stages	Dates															
	3–22	4–5	4–19	5–3	5–17	5–31	6–14	6–28	7–12	7–26	8–9	8–23	9–6	9–20	10–4	10–18	11–1
B + C + D	23.1	22.2	20.0		25.0	42.9		75.0		100	100	100	100	100	100	100	100
C + D	0	0	0		0	0		10.0		37.5	50.0	57.9	71.4	92.3	100	100	100
D	0	0	0		0	0		0		0	0	5.3	14.3	46.2	57.1	52.9	83.3
B + C + D (Adjusted*)	0	0	0		0	20.0		68.8		100	100	100	100	100	100	100	100

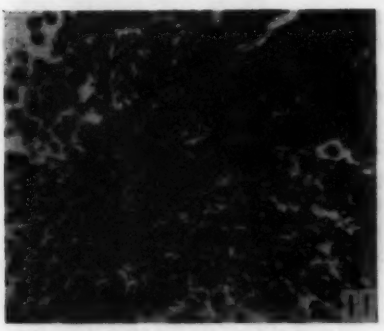
* Per cent in Stages B + C + D after subtraction of 20 per cent of sample size from Stage B and from total.











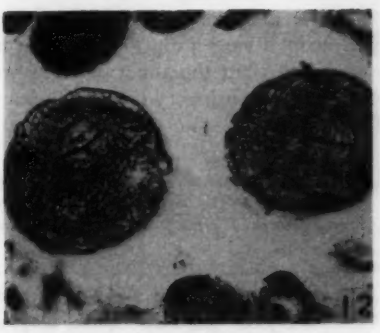


FIGURE 7. Almost empty follicles of male in advanced stages of spawning. × 112.

FIGURE 8. Female gonad in late October still containing some old ovocytes but also forming new ones to be discharged next year. × 112.

FIGURE 9. Female gonad in December showing largely small growing ovocytes, but also containing some that are morphologically ripe. × 112.

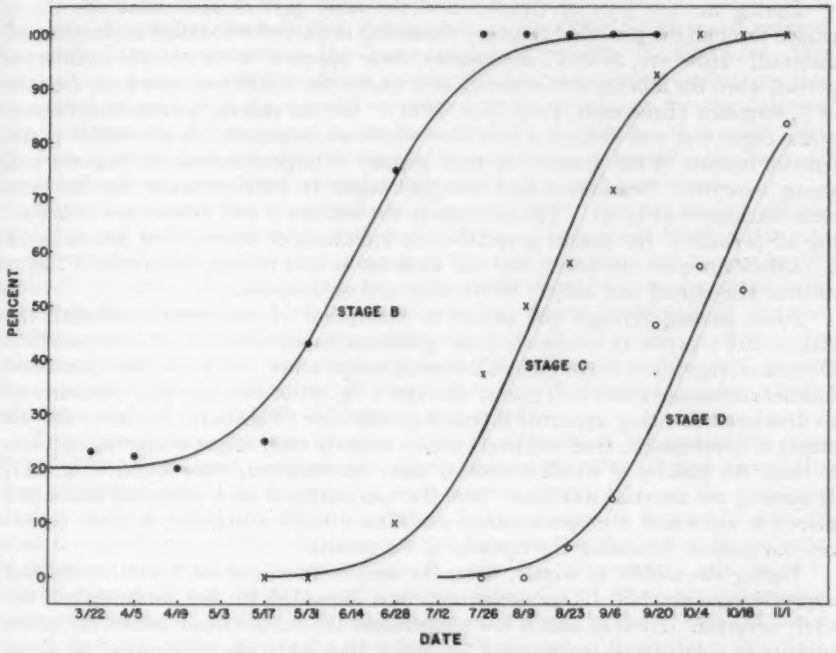


FIGURE 13. Percentage of clams in successive stages of sexual maturity at different times of the year. Stage B: ripe but had not begun to spawn; Stage C: partly spawned; Stage D: completely spawned. Additional explanation in text.

in earlier stages of development (Fig. 7). The emptied follicles were not contracted but remained distended, as was noticed for *V. mercenaria* (Loosanoff, 1937b), and in some instances they gradually became filled with vacuolated cells.

During September, when the water temperature was at its maximum of about 15.0° C., and when partially and fully spawned clams were becoming progressively more numerous, ripe males, showing the so-called "packing" arrangement of the sperm, and ripe but unspawned females still were encountered. In October the temperature of the water was slowly declining but, nevertheless, spawning continued and during the first part of the month was, perhaps, even more active than in September. By the middle of the month the majority of the animals had completed spawning, and towards the end of the month the number of clams with spawn was rapidly diminishing (Tables I and II, Fig. 13).

FIGURE 10. Morphologically ripe gonad of a female during late December or in January. × 112.

FIGURE 11. Male gonad in late December or in January already containing ripe spermatozoa. × 475.

FIGURE 12. Eggs ready to be discharged in spawning showing dissolution of the germinal vesicle and the formation of chromosomal spindle. × 475.

During the last part of October and the early part of November the clams passed through the period of recovery consisting in part of resorption of unspawned material. However, as in *V. mercenaria,* there appeared to be no true indifferent period, when the follicles are free of all cells except the indifferent ones as is the case of *C. virginica* (Loosanoff, 1942; Coe, 1943). On the contrary, even then the sex of the clams was well defined, a condition which was especially clearly visible in the females because of the presence in their gonads of large numbers of ovogonia and young ovocytes. Sometimes such ovocytes began to develop before the old ones were discharged (Fig. 8). The presence in the follicles of well defined sex cells during all periods of the annual gonadal cycle indicated, of course, that sex in adult *C. islandica* is quite stable and that sex reversal, as was recently shown for *V. mercenaria* (Loosanoff and Miller, 1950), rarely, if ever, occurs.

After passing through the period of resorption of unspawned material, the clams enter a period of extremely rapid gametogenic activities which continues into December, regardless of the rapidly declining temperature. Some females possessed follicles containing either well grown ovocytes (Fig. 9) or ovocytes that were already so developed that they appeared morphologically ripe (Fig. 10). In males also all stages of development, from relatively unripe males in early stages of spermatogenesis to those the follicles of which contained many spermatozoa, were found (Fig. 11). If some of the material was taken from the spermaries of such advanced males and placed in sea water, the spermatozoa would soon begin swimming in their typical circular motion, indicating the ripeness of the gonads.

During the middle of winter, when the temperature is at its lowest, sometimes approaching only 1.5° C., gametogenesis was slowed down but probably not entirely arrested. Even at such a low temperature the clams remain somewhat active because in a laboratory experiment, in which they were placed in water of about 1.0° C., the clams opened the shells, extended their siphons, and pumped water, thus indicating that they were not hibernating.

With a gradual increase of the temperature in the spring, gametogenesis became progressively more rapid and eventually, by the end of June or early July, a state of physiological and morphological ripeness was achieved by individual clams.

Although the observations on the seasonal gonadal changes of the clams were carried on throughout the entire year, only those data obtained between March 15 and November 1 were analyzed statistically. The late fall and winter conditions were not included in the quantitative analysis. During that period, as already pointed out in the histological discussion, the gonads went through the recovery stage followed by rapid gametogenesis as the result of which approximately 20 per cent of the clams appeared to be morphologically ripe by about March 15.

For the quantitative studies the data were grouped in intervals of two weeks, accepting the mid-point of each of these intervals as the date for all the observations of that group. There were seventeen such intervals but in three of them observations were lacking (Table I).

All clams were assigned to one of four stages depending upon gonad condition. Stage A contained clams which were generally unripe but which, nevertheless, contained some ripe cells. The clams of Stage B were ripe, at least morphologically, but had not begun to spawn. Stage C consisted of partly spawned clams, while Stage D contained those that were completely spawned.

The observed frequencies of the clams in each of the four stages are presented in Table I. Inspection showed that the percentage of clams which have entered Stages B, C or D increases as the season progresses (Table II, Fig. 13). The analysis was based on the provisional assumption that the relation underlying this increasing percentage is the cumulative normal curve, the curves for the different stages of maturity differing only in the dates of their succession. In general, Figure 13 is a comparison between the cumulative percentages obtained from Table II and the theoretical cumulative normal curves which have been postulated.

Since it was found that approximately 20 per cent of the clams possessed gonads that appeared to be morphologically ripe by March 15, the lower limit of the percentage of clams that had entered Stage B at that date was not equal to zero but to about 20 per cent. Our hypothesis was extended to allow for this percentage, only assuming the normal cumulative curve of ripening for the remaining 80 per cent of the population. Accordingly, the number of clams closest to 20 per cent of the total sample in each group was subtracted from Stage B and from the total, giving the values of adjusted percentage in Stages B + C + D (Table II). No adjustment was made in the analysis of Stages C + D and D.

The cumulative percentages given in Table II, adjusted in the case of the class B + C + D, were used to estimate the approximate dates when 50 per cent of the clam population would be expected to have entered Stages B, C or D. Analysis showed that these dates probably fall within ± 3 days of June 15, August 13 and October 6 respectively. Thus, the time interval between the date when 50 per cent of the clams are expected to enter Stage B (ripe but unspawned) and Stage C (partly spawned) was 59 days, and between Stage C and Stage D (completely spawned), 54 days. It should be emphasized once more, however, that the mean date of June 15 applies only to the ripening of 50 per cent of those clams that were not morphologically ripe by March 15. As is evident in Figure 13, the mean date for the population as a whole is about June 4.

The same analysis showed a population standard deviation of 29 days. This means, for example, that 95 per cent of the population would enter a given stage during a period extending from about 60 days before to 60 days after the mean date.

As mentioned above, the standard error of the three critical dates was estimated to be ± 3 days. Larger samples of clams should provide greater precision of these estimates, but at present such refinement does not seem necessary. It was also possible to estimate the goodness of fit of the cumulative normal model, making a separate test of the assumption of equal standard deviations for the three stages of development. After a maximum likelihood fitting by the methods of probit analysis the residual $\chi^2 = 8.68$. We have allowed 28 degrees of freedom in the original 4×14 table, disregarding those classes with an expected frequency of less than one clam. In fitting the curves given in Figure 13, 18 constants have been used: 14 sample sizes, 3 means and the standard deviation. This leaves 10 degrees of freedom for χ^2, showing a satisfactory agreement. If individual slopes are fitted for the three cumulative curves, the sum of the 3 residual χ^2's = 6.77 with 8 degrees of freedom. The difference, $\chi^2 = 1.91$ with two degrees of freedom, arises from lack of parallelness of the three lines, and is not significant.

Our attempts to induce spawning in ripe *C. islandica* always failed. Methods, such as rapid increase in temperature, addition of suspension of sex products,

changes of pH, salinity, etc., produced no results. During the spawning experiments it was found that the clams do not tolerate well temperatures over 27.0° and that at temperatures of 30.0° C. or higher they looked as if anesthetized, a condition manifested by relaxation of the adductor muscles and by expansion of the foot. Usually, subjection to such a high temperature was followed within a few days by a heavy, usually complete, mortality.

Natural unprovoked spawning was observed in only two cases. Once it occurred in the middle of winter, on February 3, 1945, when a single female kept in an aquarium in a cold room, the temperature of which was increased by direct sun to only 9.0° C., discharged a large number of eggs. The actual act of spawning was not seen but the eggs were found in a mass lying next to the clam. The eggs ranged from approximately 80 to 95 μ in diameter.

The second spawning occurred on July 19, 1948 when clams of both sexes, used several days prior to that date in an unsuccessful spawning experiment, spawned during the night. The temperature at the time of spawning was 22.0° C. The size of the eggs varied between 85 and 90 μ. Some of the eggs were fertilized and showed normal early development.

Attempts to fertilize artificially the eggs stripped from ripe females always failed because such eggs, even after being placed in sea water, still retained intact their germinal vesicles. Under normal conditions the germinal vesicle dissolves while the egg is still in the gonads of the mother clam, just before being discharged in the process of spawning. Immediately upon dissolution of the germinal vesicle a chromosomal spindle is formed (Fig. 12). After that the egg is ready for fertilization. In the stripped eggs, however, such changes do not occur and, therefore, fertilization is impossible. In this way *C. islandica* again closely resembles *V. mercenaria* in which artificial fertilization also cannot be achieved.

In preparing this article I feel especially obliged to my colleague, David W. Calhoun, for his statistical analysis of the data. I also wish to extend my sincere thanks to Charles A. Nomejko for the preparation of the photomicrographs and to William Arcisz for collecting and shipping to me many samples of clam gonads on which this article is based.

Summary

1. The main period of gametogenesis in both sexes of *Cyprina islandica* occurs in late fall and early winter. In December or January the gonads of some clams present a morphologically ripe appearance, such individuals constituting approximately 20 per cent by March 15. Gametogenesis is slowed down but not entirely arrested during late winter and is resumed at a rapid rate with the spring increase in temperature.

2. Spawning begins near the end of June or early in July when the water temperature is approximately 13.5° C.

3. The dates when 50 per cent of the clam population should be expected to enter the stage of being ripe but not having begun to spawn, the stage of partial spawning, and the stage of completed spawning should fall within ± 3 days of June 4, August 13 and October 6 respectively.

4. In adult *C. islandica* the sexes are separate and well defined even immediately after spawning, thus indicating that sex reversal is uncommon.

5. The eggs of *C. islandica* cannot be artificially fertilized.

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ACTIVITY IN A CRUSTACEAN GANGLION. I. CARDIO-INHIBITION AND ACCELERATION IN PANULIRUS ARGUS[1]

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The cardiac nerves of arthropods have been studied from time to time (Krijgsman, 1952), but there has been no extended work on the decapod Crustacea. Much has been done on *Limulus* (Carlson, 1905, 1905–06; Heinbecker, 1933, 1936; Garrey, 1942; Prosser, 1943) which seems to have an analogous system with respect to cardiac ganglion and extrinsic nerves, but the ganglion contains a number of cells and by its consequent complexity is of limited usefulness in determining the mechanism of its action as a pacemaker for the heart beat and its relation to the central nervous system.

The decapods have a relatively simple ganglion, or one containing a limited number of cells (Alexandrowicz, 1932) and relatively few extrinsic fibers connecting the ganglion with the central nervous system. The normal heart beat has been shown to have its origin in a burst of nervous activity in the cardiac ganglion (Welsh and Maynard, 1951). A study of some of the details of this activity has been made and will be presented in another paper. Briefly, the frequency of beat is determined by the frequency of the bursts of nervous activity, and the amplitude of contraction is determined by the number of motor impulses per beat. Each cell, as in *Limulus* (Prosser, 1943), may "fire" a number of times per burst.

The heart beat may be augmented or inhibited, both in amplitude and rate, if the proper extrinsic nerves connecting the ganglion with the central nervous system are stimulated. Wiersma and Novitski (1942) give an excellent account of the qualitative effects of the extrinsic nerves in the crayfish heart, and Smith (1947) studied the crab heart and the pharmacology of the extrinsic nerves. However, no quantitative study of the effects of these nerves has been published. The present investigation was undertaken to determine the effects of varying parameters of stimulation of each of the extrinsic nerves. The cardio-inhibitor is of particular interest, for if its action is upon the ganglion, as seems to be the case, this is a preparation in which an inhibition, possibly analogous to central inhibition, may be studied. The pertinent anatomy was worked out, and a general description, supplementing that of Alexandrowicz (1932), is given.

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Materials and Methods

Thirty-eight adult *Panulirus argus* (Latreille), the Bermuda spiny lobster, were used. Antennae and legs of the lobsters were removed, the ventral nerve cord severed at the base of the abdomen to prevent movement, and the animal allowed to bleed. The carapace anterior to the cervical groove was then removed, and the viscera dissected out. Care was taken to prevent leakage of digestive fluids into the cavity, which was washed with sea water upon removal of the organs. The heart was perfused with sea water by means of a cannula inserted into the opening of the ophthalmic artery. After a period of initial irregularity, the heart usually beat regularly and continuously for several hours at room temperature, about 27° C.

For morphological study, the heart or portions of the central nervous system were stained with dilute methylene blue in sea water. The preparation was usually studied fresh, and then fixed in ammonium molybdate (Alexandrowicz, 1932).

In such a preparation, the cardio-inhibitors are exposed on the surface of the abdominal flexors; the first and second cardio-accelerators may be exposed by cutting and lifting the first and second abdominal flexors. The accelerators are also visible for a short distance laterally above the muscles before entering the pericardium.

Silver wire hook electrodes 2.5 mm. apart were used for stimulation. Square stimulus pulses were supplied by a Grass 3-BC stimulator.

The heart contraction was recorded by kymograph. The recording lever was attached to the cannula perfusing the heart rather than to the heart directly.

The percentage of acceleration or inhibition was obtained by dividing the change in beat by the normal rate determined immediately before stimulation. The heart rate was not constant, usually varying plus or minus 10% during a run of several minutes and some 20% over several hours. The average maximum and minimum rates recorded in the normal beat of 11 animals were 71.2 and 47.8 beats per minute. The absolute maximal and minimal rates for these animals were 115 and 29.5 beats per minute. In general, a difference of 10% is significant in determining the effects of the extrinsic nerves.

Results

Anatomy

The cardiac ganglion (Alexandrowicz, 1932; Welsh, 1939) is similar to those of other marine decapod Crustacea. It is Y-shaped and lies on the inside of the dorsal wall of the heart, crossed by one or two muscle strands. There are five large ganglion cells; four of these are grouped anteriorly, close together at the fork of the "Y", the fifth occurs about half-way down the stem at the junction of prominent side branches. In the posterior half of the stem, there are four smaller cell bodies arranged linearly with more or less equal spacing. The large cell bodies averaged $54 \times 84\,\mu$ in fixed preparations, and the small cells, $22 \times 43\,\mu$. Fibers from the ganglion cells first run in the central trunk of the ganglion and then branch into the myocardium, ramifying throughout the muscle. They also give off collaterals which form neuropiles within the trunk or extend into the muscle to form arborizations (see Alexandrowicz, 1932).

The cardiac ganglion is connected to the central nervous system by fibers which arise in the thoracic ganglion and reach the heart via the inhibitor or accelerator nerves, the pericardial plexus, and the dorsal nerve (Fig. 1A).

The pair of cardio-inhibitor nerves (CI) arises in the anterior region of the thoracic ganglion. On each side, one of the pair runs forward along the sternal canal to the cephalic apodeme, where it leaves the canal, passes through a small opening or notch in the endophragmal plate, and then runs dorsally along the surface of the abdominal flexors to the anterior border of the pericardium. There it enters the pericardium and joins the lateral pericardial plexus (pp).

The two pairs of cardio-accelerators (CA-1 and CA-2) arise immediately posterior to the cardio-inhibitor in the ganglion. The second is the most anterior of a pair of nerves arising from the base of the nerve to the first pereiopod; the first accelerator is anterior to this, presumably from the region at the base of the third maxilliped nerve. These would correspond to segmental nerves "m" and "l" which were described by Heath (1941) in the crab, *Pugettia*. They run forward along the sternal canal for a short way, but leave it through sinuses in the endophragmal plate in the first and second segmental region, respectively, behind the cardio-inhibitor. Then they run dorsally beneath the abdominal flexors to the pericardium where they enter the lateral pericardial plexus. The posterior cardio-accelerator branches before entering the pericardium, its anterior branch going to the plexus while the posterior branch proceeds to the pericardium sending off twigs to the muscles of the dorsal region and to the ligaments of the heart.

A pair of dorsal nerves (DN) carries the accelerator and inhibitor fibers from the lateral pericardial plexus to the heart. On leaving the plexus, one of the pair may contain as many as 11 methylene blue-stainable fibers; one of these branches off soon after leaving the plexus and enters the dorsal musculature. The others remain until the nerve reaches the lateral suspensory ligaments where the nerve passes through another plexus which ramifies over the ligaments. Here most of the fibers in the dorsal nerve branch and leave until two to four are left which actually enter the heart. These fibers can be traced back through the ligamental plexus (lp) and shown to proceed directly along the nerve from the lateral plexus. They may be considered as cardio-regulators, while the additional number originally in the dorsal nerve appear to be concerned with some function in the ligaments. As shown in Figure 1A, the ligamental plexus is composed of ramifications of branches from the lateral plexus. The posterior ligamental plexus may also receive branches from the posterior branch of the second cardio-accelerator.

The dorsal nerve enters the heart dorso-laterally between the dorsal ostia (Fig. 1A). After a slight swelling at the surface of the myocardium, it usually runs directly to the ganglionic trunk at the level of the third or fourth large cell. There is much variation; the path of the nerve on each side of the heart is usually different, and in some cases small branches are given off to the muscle or there is an anastomosis with nerve branches from the central trunk. It could not be ascertained whether the small branches contain collaterals from the regulator fibers. In the anastomoses, there was no union of the actual nerve fibers; the sheath of connective tissue is merely united.

Alexandrowicz (1932) distinguished thick and thin fibers within the dorsal nerve which he termed System I and System II. In the dorsal nerve of *Panulirus*, a maximum of four fibers, similar in size (5 to 9μ), stained well with methylene

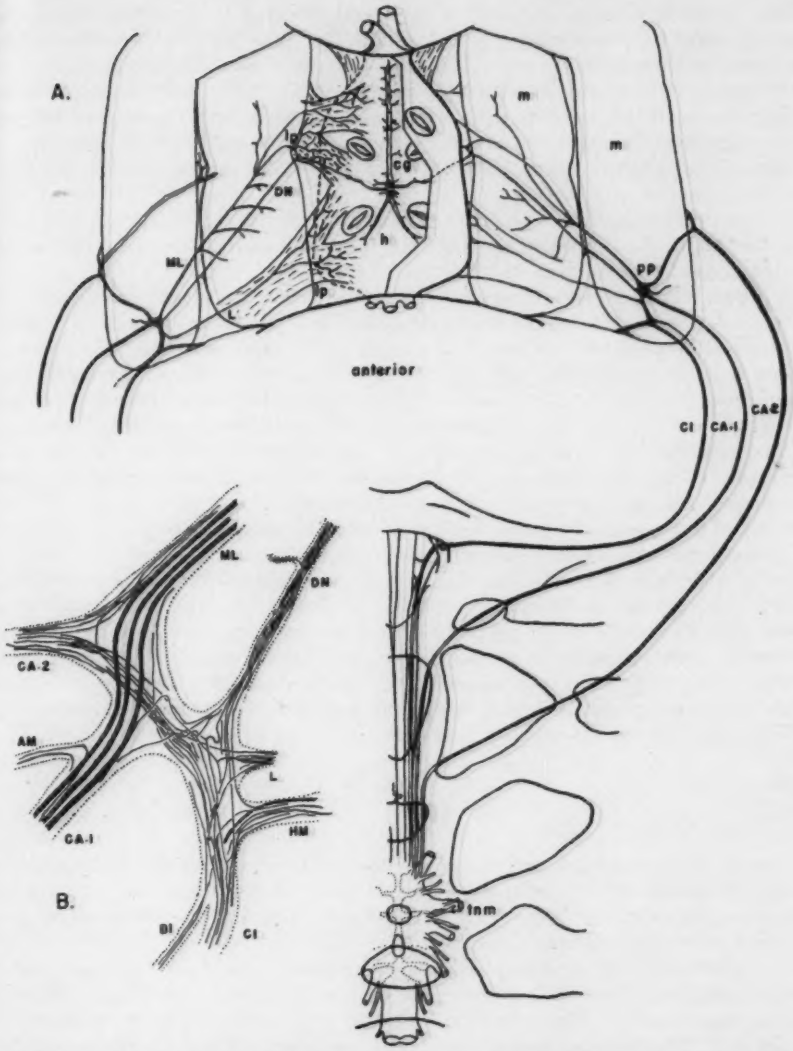

FIGURE 1. Cardiac nerves of *Panulirus argus*. A. Thorax split horizontally and upper-half laid back, giving dorsal view of thoracic ganglion and ventral view of heart. The heart is slightly enlarged with ½ ventral side removed to show cardiac ganglion. B. Left lateral pericardial plexus, enlarged to show nerve fibers. AM, branch to dorsal abdominal muscles; BI, branch from cardio-inhibitor; CA-1, first cardio-accelerator; CA-2, second cardio-accelerator; cg, cardiac ganglion; CI, cardio-inhibitor; DN, dorsal nerve; h, heart; HM, nerve to hypodermis and dorsal muscles; L, ligamental nerve; lp, ligamental plexus; m, muscles; ML, nerve to ligaments and dorsal muscles; pp, lateral pericardial plexus; tnm, thoracic ganglion.

blue. These correspond to System I. Upon entering the ganglion they divide, sending equal processes anteriorly and posteriorly in the trunk. These immediately send out small branches which either form networks over the surface of the large cells or help form neuropiles within the ganglion. The main processes can be traced along the trunk and seen to send out occasional branches. According to Alexandrowicz, such branches end in the muscle close to the endings of the ganglion cell fibers. The regulator fibers would therefore act both in the ganglion on cell bodies and in the muscle.

System II stained only in rare instances when smaller fibers were noted entering the ganglion with System I. They may have also been present but unidentifiable in the general fiber complex of the trunk.

Figure 1B gives semi-diagrammatically the general relationships of the nerve fibers entering and leaving the lateral plexus. There are about nine nerves involved: 1) the cardio-inhibitor, 2) a branch at the base of the cardio-inhibitor which proceeds along it for a distance (BI), 3) the first cardio-accelerator, 4) a branch containing two fibers from the first cardio-accelerator which runs to the abdominal extensor (AM), 5) the second cardio-accelerator, 6) a branch which contains large motor fibers from the first cardio-accelerator and smaller fibers supplying the suspensory ligaments of the heart (ML), 7) the dorsal nerve, 8) the anterior ligamental nerve, running to the anterior suspensory ligaments of the heart, 9) a branch consisting largely of fibers from the cardio-inhibitor which do not cross the plexus but appear to run dorsally into the muscles and hypodermis (HM). The nerve paths may vary greatly, especially (6), which as 'ml' in Figure 1 follows a different course on each side. The number of fibers shown in Figure 1B is not complete, for all did not stain with methylene blue. A number of fibers entering the dorsal nerve branch before entering, while there are some from each of the cardio-accelerators and the cardio-inhibitor which do not branch. It is among these latter that the actual regulator fibers are probably found, while the branching fibers are presumably those which innervate the ligaments of the heart.

Physiology

1. *Cardio-inhibitor*

Both the number of active fibers and the frequency or pattern of impulses per fiber presumably act in producing a graded effect on the heart beat. In analysis, therefore, the response of the nerve to stimulation was separated from the response of the heart to the inhibitor nerve.

a. Excitability of inhibitory fibers. A frequency was chosen (70 per second) which had been shown to give complete or nearly complete inhibition. This was kept constant and the threshold at varying strengths and pulse durations determined (Fig. 2). The pseudochronaxie was 0.2–0.5 milliseconds, and k value (Hill's time constant), 0.29–0.72. In one case, not in Figure 2, a pseudochronaxie of 1.45 milliseconds was obtained.

The absolute values of the pseudochronaxie, though subject to errors due to polarization of the electrodes, are of the same order of magnitude as others obtained for crustacean nerves (Jasper and Monnier, 1933). The lack of irregularities in the curve indicates that a single physiological unit is being stimulated. At threshhold

voltage, the response was often merely a temporary hesitancy in the heart beat, indicating a transitory stimulation at the electrodes.

If either voltage or pulse duration was well above minimum threshold value, increasing the other parameter caused a stepwise, not graded, increase in inhibition. Thus, an incompletely inhibited heart showed no effect as the pulse duration was increased several hundred per cent. Then suddenly a second threshold level was reached and the heart became completely inhibited. Usually there were two such levels, though in one case there were three, and in several instances there was no increase over the first minimum threshold effect. If either parameter was near minimum threshold value, the second level could not be obtained (Fig. 4). It thus has the qualities of a true second threshold.

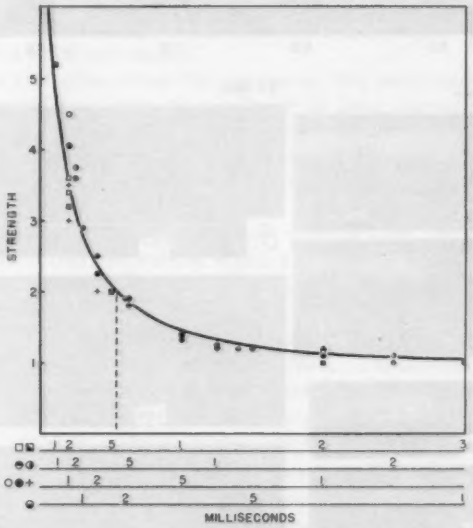

FIGURE 2. Strength-duration curve. Ordinate in multiples of rheobase. ○, cardio-inhibitor; □, first cardio-accelerator; +, second cardio-accelerator.

With increasing voltage and a constant pulse duration, there was a small but definite range (0.05–0.2 volts) at minimum threshold and at each step where there was a gradation before the final level of inhibition was reached. It would appear that in this relatively narrow range the fiber responds to some but not all of the stimuli, but that after the fiber responds to each stimulus, a change in voltage no longer causes increased response in that fiber.

In several instances it was apparent that the fibers running to either the ligamental plexuses or the dorsal musculature were stimulated, for a change in the base line not otherwise associated with the heart beat was obtained upon stimulation.

With possible exceptions at low frequencies of stimulation, repetitive responses to each stimulus did not occur. The regular relation between frequency and effect,

the fact that changing pulse duration and voltage did not result in a consequent gradation in effect, and the low pseudochronaxies argue for this conclusion.

Exhaustion of the cardio-inhibitor was noted in cases of long-continued stimulation. There was either a gradual and irregular, or sudden increase in the heart

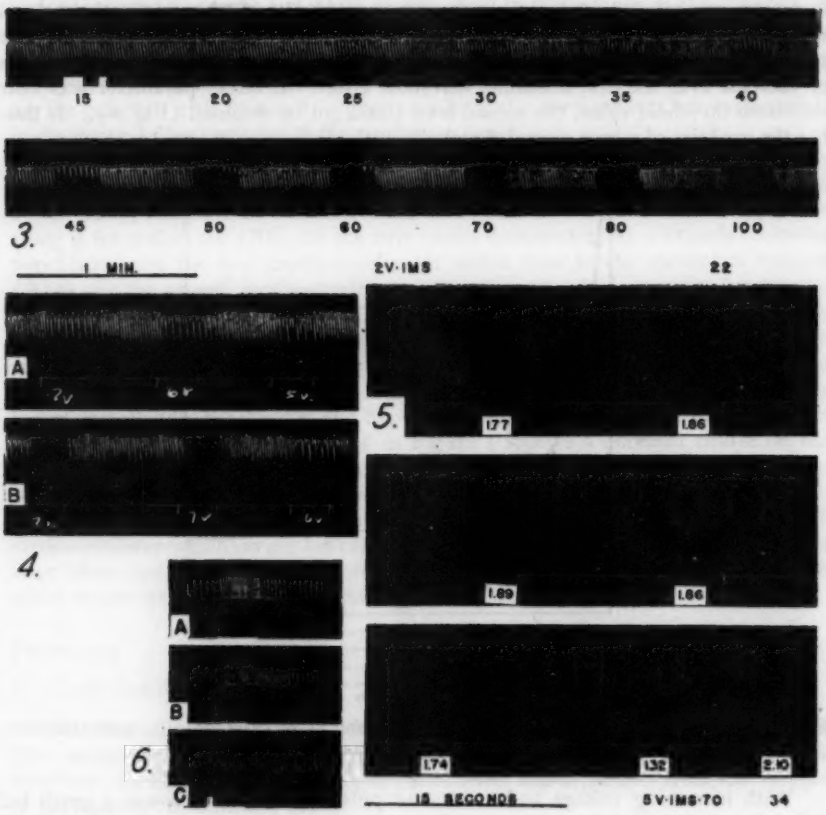

FIGURE 3. Cardio-inhibitor. Frequency varied; stimulus strength, 2 volts, 1 millisecond pulse duration.

FIGURE 4. Cardio-inhibitor; interrelation of pulse duration and stimulus strength. Stimulus frequency, 40 per second, voltage varied. A. Pulse duration, 0.2 milliseconds; B. pulse duration, 0.5 milliseconds. Note alternating beat.

FIGURE 5. Cardio-inhibitor. There is progressively less effect on the beat immediately following the initiation of stimulation as the time between these events is decreased. Stimulation at 70 per second, 5 volts, 1 millisecond.

FIGURE 6. Cardio-accelerator. A. Cardio-accelerator 1; stimulation at 50 per second, 1.4 volts. B. Cardio-accelerator 2; stimulation at 50 per second, 0.5 volts. Note the after-effect. C. Cardio-accelerator 2; stimulation at 15 per second, 5 volts. The alternating beat is abolished upon stimulation, but returns upon the release of stimulus.

beat. Since it was found that the recovery process following accomodation or exhaustion of the cardio-inhibitor was slower or followed a different time course from the accomodation itself (Levin, 1927), a 15-second stimulus period was usually followed by a 30-second rest in determining the stimulus-response curve. This in most cases proved adequate to maintain the nerve in an unexhausted state.

b. Response of the heart to the inhibitor. At a given level the percentage of inhibition obtained was characteristically a non-linear function of the frequency of stimulation. In most cases complete inhibition was obtained by stimuli at 100 per second. Exhaustion of the nerve was frequent at frequencies between 80 and 500, and the threshold of stimulation was often raised (Pantin, 1936). The upper limit of the frequency of stimulation appears, when obtained, to be due to the failure of the cardio-inhibitor rather than to some effect at the cardiac ganglion. The minimum frequency producing detectable inhibition was about 10–15 per second (Fig. 3). In several instances stimulation at 15 per second or less had slightly more effect than stimulation at 20 per second.

As the frequency of stimulation was increased, the percentage of inhibition, as measured by decreased frequency or amplitude of beat, increased. Because each of the two or three threshold levels gave a different stimulus-response curve, it was impossible to average the results of all experiments. Values obtained for six animals were grouped roughly according to the pulse duration of the applied stimulus (Fig. 7). Curve 1 is above the first threshold, the pulse duration is rather short. In Curve 2, the second threshold level, the pulses were of long duration, and in Curve 3 are the long pulses of one preparation which also showed threshold levels 1 and 2. With aging the thresholds tended to change, and in several instances the older preparations showed only the first threshold effect.

Usually the inhibited amplitude and frequency remained regular and in some instances served to regularize a normally irregular beat. Occasionally, however, stimulation of the inhibitor caused an irregular, inhibited beat. Since this irregularity was also noted near the nerve threshold, it seems most likely that it was because of the irregularity of the impulses arriving at the ganglion. In certain other instances a beat alternating between a strong and weak contraction (Fig. 4) was initiated. This seems to be a peculiarity of the ganglionic action and was very often noted in old or slightly injured preparations. It is also found in non-nervous but rhythmically active centers such as the molluscan heart (Welsh and Taub, 1950, Fig. 1). Near the frequencies causing complete inhibition it was not unusual to find the heart contractions either quite arrhythmical or of varying strengths. It is not clear whether these, and the occasional escape beats which occurred during otherwise complete inhibition, are due to changes in the threshold of the cardio-inhibitor nerves and consequently the number of impulses arriving at the ganglion, or whether they are due to some change in the threshold of the ganglion's response to the arriving impulses.

Facilitation is present, but the period of facilitation must be quite short. At frequencies of 20 per second, there is some evidence that there is a progressive inhibition during the first few beats. At higher frequencies the heart beat is much too slow to indicate facilitation in the ganglion, and inhibition is immediate. As shown below, a stimulus of 70 per second may produce its maximum effect in less than ⅓ of a second.

If, in a heart beating approximately every two seconds (Fig. 5), a stimulus of 70 per second was applied up to 1.7 seconds after a beat, the next beat was completely inhibited. If, however, the stimulus was applied between 1.7 and 2.1 seconds after a beat, the following heart contraction was only partially inhibited. Thus for a period of ⅓ second, a progressive inhibition in the beat following the initiation of the stimulus was obtained. It would seem most probable that this is accomplished by blocking a portion of the nervous activity in the ganglion, and that the ⅓ second measures the maximum duration of facilitation at this frequency of stimulation.

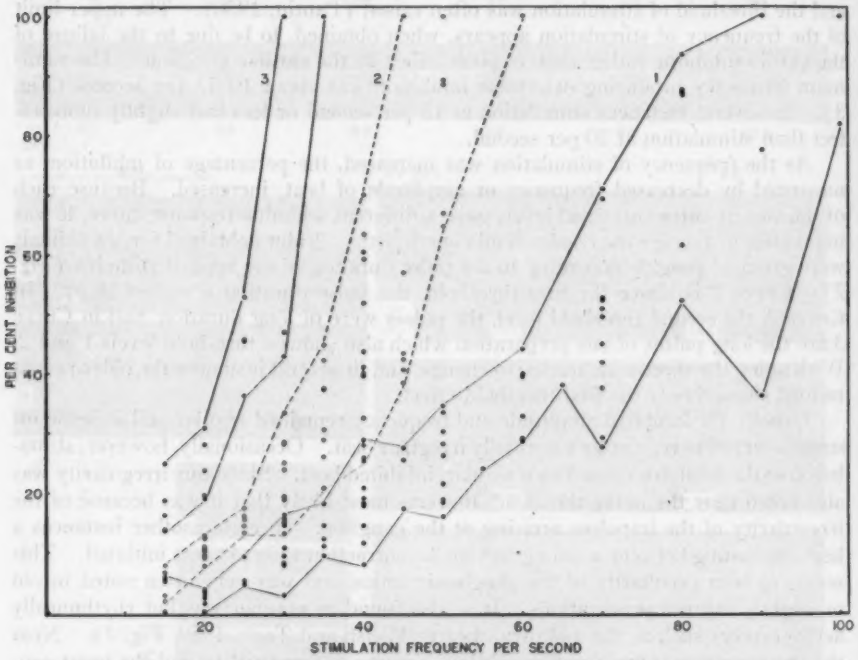

FIGURE 7. Cardio-inhibitor; stimulus-response curves for 6 animals. Curve 1, pulse duration, 0.2 to 5 milliseconds; Curve 2, 1 to 10 milliseconds; Curve 3, 5 to 10 milliseconds. In any one animal there was a sharp division between curves, but the thresholds varied among animals.

Four phenomena may be noted upon cessation of inhibitory stimulation: 1) there may be a series of beats of gradually increasing frequency and amplitude until the normal state is reached; 2) there may be an exceptionally large contraction immediately following the release of inhibition (this may be followed by a period of contractions alternating in amplitude and frequency, or a gradual increase as in (1)); 3) there may be a period of accelerated beat and larger amplitude immediately following inhibition; 4) there may occasionally be no noticeable after-effect. The duration of inhibition does not greatly influence the after-effect.

2. Cardio-accelerators

The investigation of the cardio-accelerators was not as extensive as that of the cardio-inhibitor. The pseudochronaxies of both cardio-accelerator nerves (Fig. 2) were not significantly different from those obtained for the cardio-inhibitor. A second threshold was found, though not as commonly as with the inhibitor. Comparisons of the thresholds at different frequencies were not made.

From the responses in Figure 6 and the similarity of the shape of the frequency-acceleration curves, it seems fairly clear that the two cardio-accelerators act in the same manner on the heart, though it is not possible to say that they have the same quantitative effect. In Figure 9A twice as many fibers were probably stimulated in the first cardio-accelerator as in the second. A later series of stimuli applied to

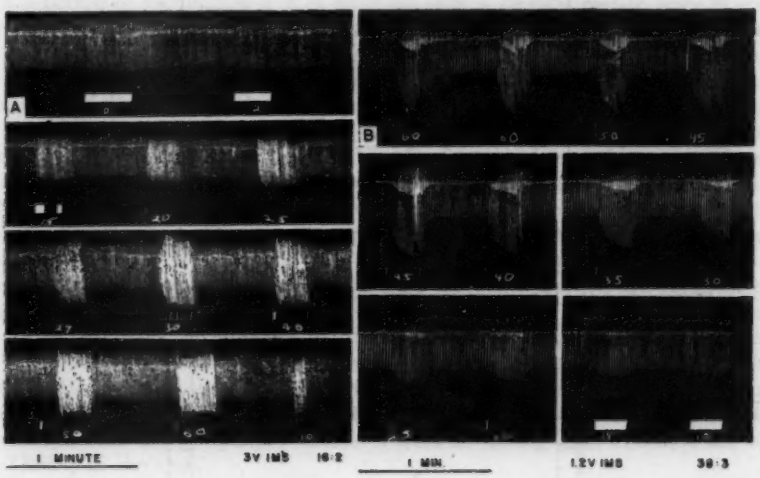

FIGURE 8. Cardio-accelerator; frequency varied. A. Cardio-accelerator 1; stimulation at 3 volts, 1 millisecond. B. Cardio-accelerator 2; stimulation at 1.2 volts, 1 millisecond.

the first cardio-accelerator produced a curve of acceleration with a maximum reduced to approximately that of the second accelerator.

In several instances stimulation of the cardio-accelerators restored a regular beat to an irregular or inactive heart. In such cases, the maximum rate obtained (95–125 per minute) was comparable to the maximum rate obtained from a normally beating heart upon stimulation (74–140 per minute). As Wiersma and Novitski (1942) found, however, the maximum was in many cases correlated with the normal heart rate. The cardio-accelerators usually destroyed an alternating rhythm by equalizing succeeding beats, redistributing a given number of impulses into equal bursts rather than by only increasing the number of impulses in the weaker bursts (Fig. 6).

The effects of varying frequencies of stimulation are shown in Figures 8 and 9.

The amplitude and rate of beat increased in a similar fashion, and there is a maximum effect which is usually reached by stimulation frequencies of 60 per second. No further effect may be obtained with frequencies up to 500 per second. The lower limit of effective frequency varied in the vicinity of 2–5 per second. In a manner similar to stimulation of the cardio-inhibitor, stimuli at 10 per second often

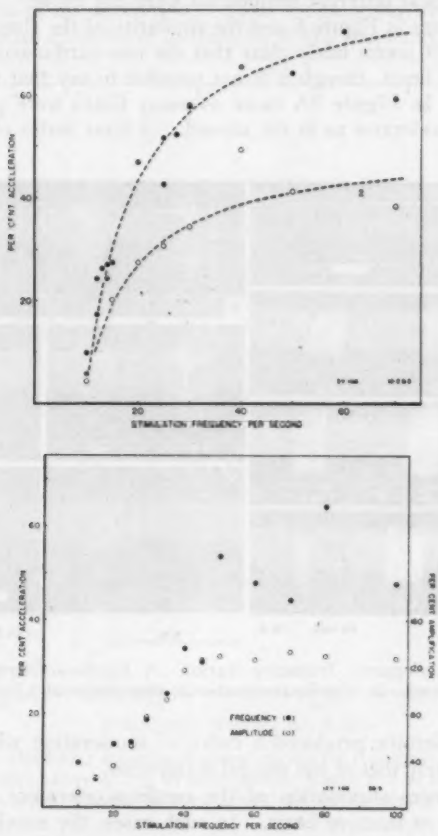

FIGURE 9. Cardio-accelerator; stimulus-response curves. A. ●, cardio-accelerator 1; ○, cardio-accelerator 2. Plot from individual of Figure 8A. B. Cardio-accelerator 2; the response curves are similar in shape for both frequency and amplitude. Plot of individual of Figure 8B.

were more effective than stimuli at 15 per second. It is not clear whether these responses at the lower frequencies involved repetitive discharges in the nerve.

The stimulus-response curves were usually hyperbolic (Fig. 9A), but in several instances a roughly sigmoid curve (Fig. 9B) was obtained. It was found im-

possible to obtain averages of several runs, for the curves varied with time and the individual.

Facilitation of the accelerating mechanism was much slower than of the inhibitor mechanism. It involved a gradual increase in both frequency and amplitude, though the latter effect was usually the more apparent. The slope of this curve, and consequently the duration of facilitation at the maximum response, was related to the frequency of stimulation. In some preparations it was not unusual to find that at lower frequencies (40 per second) facilitation was too rapid to be noted by the amplitude of the heart beat (Fig. 8). In fact, often the first few beats af a period of stimulation were of super-amplitude, caused apparently by the sudden high degree of excitation applied to the pacemaker which destroyed momentarily the equilibrium presumed necessary for a rhythmic beat. This could also be considered a period of facilitation if it is assumed that a slightly higher level of excitation is necessary to restore regularity at increased rate and amplitude. In some cases where the frequency of stimulation was fairly low (25 per second) there was a slow linear increase in amplitude after the first period of rapid facilitation.

The effects following the release of stimulation can be grouped into three classes. 1) There was an exponential decrease in rate and amplitude, bginning immediately or soon after the release of stimulation. Usually the duration of this was not over 10 seconds, though the final amplitude after stimulation may remain slightly greater than that before stimulation. 2) In three cases the exponential decrease fell to a level of subnormal amplitude and frequency which was followed by a gradual return to the normal beat. In the case of amplitude, this may last for nearly a minute. This effect was especially noted at high frequencies, when low frequencies yielded effect 1, and was to some extent dependent upon the duration of stimulation. 3) In two cases the stoppage of stimulation had very slight or an increased effect. The latter case must have been caused by after-discharge in the nerve. In the former, there was a sudden dip in amplitude upon the release of the stimulus followed by a slow return to normal amplitude lasting well over a minute. The rate returned to normal more rapidly. This is similar to lasting after-effects noted by Smith (1947) in the crab.

Discussion

The System I fibers in the dorsal nerve are probably inhibitory. Their number corresponds well with the number of thresholds, and they make intimate connection with the large cell bodies and neuropiles within the ganglion. The homologous fiber in the stomatopods is inhibitory (Alexandrowicz, 1934). No evidence was obtained concerning the function of System II.

The several thresholds are probably best explained by assuming that new cardioregulator fibers were recruited with increasing stimulus strength. The stepwise increase in inhibition, as mentioned above, can thus be correlated with the limited number of fibers observed (2–4). Likewise the all-or-none type of response at each threshold is as expected if a limited number of fibers of different thresholds were stimulated with a continuously increasing stimulus strength.

Assuming the different thresholds indicate new fibers stimulated, both temporal and spatial summation are clearly shown, especially in the inhibitor. There, temporal summation is slightly more efficient than spatial. Both, however, follow essentially the same "exponential" stimulus-response curve.

Though the stimulus-response curves are quite different from a mathematical viewpoint, they probably do not indicate radically different physiological processes. As shown by Rosenblueth and Rioch (1933) in discussing the response curves of mammalian neuro-effector junctions, the change from an apparent hyperbola to a sigmoid need not indicate a change in the process. Rather, the relative rate of recovery of a preparation following a single nerve impulse, or, as they assumed the rate of destruction of a mediator, in relation to the frequency of stimulation could determine whether a sigmoid or a hyperbola would be obtained. Thus the sigmoid frequency-acceleration curve would indicate that the recovery following each impulse was more rapid in this preparation than in one which produced a hyperbolic curve. The inhibitor stimulus-response curve could also fit here, in which case it would correspond to the lower portion of a sigmoid. Complete inhibition would generally be reached before the flexion point. The short after-effect of inhibition as compared with acceleration is evidence for the required rapid recovery.

These curves, however, do not indicate whether the process is via the release of a chemical mediator as Rosenblueth and Rioch felt. It seems possible that an electrotonic potential, or some other effect, built up by a series of pulses could be the inhibiting or accelerating stimulus.

In any case, if an inhibiting mediator is present, it is probably not that noted by Parrot (1941) in the blood stream after inhibitory stimulation, which caused activity in the intestine, for this was obviously not rapidly destroyed.

The alternating rhythm deserves some mention. In the normal heart beat, a burst of nervous activity is followed by a silent period and then another burst (unpublished observation). The continuity of this regular action is assumed to depend on a balance between activity and recovery of all the cells of the ganglion. Under some conditions the excitability states of the ganglion cells get out of phase. A burst of activity may be just great enough to prevent all the cells from recovering completely by the time the next burst is initiated by the most rapidly recovering cell. Therefore, the second burst is smaller, some of the cells "fire" fewer times than before, and the following recovery period is shorter, for the interval seems dependent upon the activity during the burst. In the next burst, the number of cells completely recovered is greater. Therefore, there is a large burst that is followed by a smaller, and so on. If the excitability of the out-of-phase cells can be either raised or lowered, a regular beat should be re-established. As a corollary, if the excitabilities of some of the cells of a regularly beating heart are either raised or lowered more than of other cells, throwing them out of phase, the alternating beat should be established. Both of the above phenomena were observed upon stimulation of the extrinsic nerves. Usually the accelerators stopped the alternating beat, though in some instances a temporary alternation was begun in an otherwise regular beat. The inhibitors generally brought about the alternation, though at times they also served to stabilize. If the above is correct, we may, therefore, conclude that the extrinsic nerves appear to affect the excitabilities of the individual ganglion cells.

I wish to acknowledge the aid of Dr. John H. Welsh in formulating this problem and the cooperation of Dr. L. Hutchins and Dr. W. Sutcliffe at the Bermuda Station. I also wish to express appreciation to Dr. R. I. Smith and Dr. T. H. Bullock for criticizing the manuscript.

Summary

The morphology and aspects of the physiology of the cardiac ganglion and the extrinsic cardiac nerves of *Panulirus argus* have been studied.

1. The extrinsic nerves consist of two pairs of cardio-accelerators and one pair of cardio-inhibitors which arise in the anterior portion of the thoracic ganglion. These run dorsally and laterally to join at the pericardial plexus where fibers from each come together to form the dorsal nerve. This runs to the cardiac ganglion. The thick fibers (2–4) of the dorsal nerve which ramify over the large cells of the ganglion are probably inhibitors. The identification of the accelerator fibers remains unclear.

2. Upon electrical stimulation of the extrinsic nerves, two or three levels of response were obtained with increasing pulse duration and voltage. This was probably because of an increase in the number of fibers stimulated.

3. Complete inhibition was usually obtained by frequencies of 35, 50 or 90 stimuli per second, depending upon the level of response of the heart. Usually no inhibition was obtained below a frequency of 15 per second. Between the minimum and maximum there was a gradation of both frequency and amplitude of heart beat. The stimulus-response curves approximated an exponential curve. Facilitation and an after-effect were present but of short duration.

4. The maximum increase in frequency and amplitude of the heart beat was usually obtained by stimulation of the accelerators at 60 per second or more. No response was ordinarily obtained below 5–10 stimuli per second. The stimulus-response curve was usually hyperbolic, though in some cases it was sigmoid. The actions of the two accelerators appear to be identical. Facilitation and after-effect have a much longer time constant than in the case of the inhibitor.

5. From a consideration of the participation of individual cells in the cardiac ganglion discharge for each beat, it is probable that the cardio-inhibitor and accelerators depress or enhance the excitabilities of the ganglion cells.

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RATE OF WATER PROPULSION IN MYTILUS CALIFORNIANUS AS A FUNCTION OF LATITUDE

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Although extensive studies have been made on the physiological effects of temperature change in poikilotherms, very seldom has this information been applied to a study of animal activity in relation to latitude. It is common knowledge that a lowering of temperature results in a reduction in metabolism in cold-blooded animals. Consequently, it is classically assumed that marine invertebrates inhabiting the colder waters of the higher latitudes have lower metabolic rates (Wimpenny, 1941).

However, there are many indications in the literature that species living in warmer and in colder latitudes are adapted in some degree to the temperature difference, not only in their tolerable limits but also in activity. For example, Takatsuki (1928) found that a species of oyster from Japan has a higher heart rate at any given temperature than another species from the tropical Pacific. Even within the same species, populations at higher latitudes are more active than those from lower at any temperature between the zones of heat depression and cold depression (Mayer, 1914 on *Aurelia;* Spärck, 1936 on several pelecypods including *Mytilus edulis;* Fox, 1936, 1938, 1939; Fox and Wingfield, 1937 and Wingfield, 1939 on polychaetes, crustacea and other groups). The result appears to be that at their natural temperatures, activity is nearly the same (Thorson, 1946, 1950 on pelecypod respiration and on the duration of larval life).

The review of Bullock (1951) covers the available information relating to temperature compensation in aquatic poikilotherms. Though the fact of the larger size of northern animals was known and the effect of increasing weight on the metabolism per unit weight of the organism was recognized, most of the earlier experimenters did not make quantitative evaluation of these factors in discussing their results. The present investigation is an attempt to understand the effect of temperature on an index of activity, the rate of water propulsion in representatives of a single species, *Mytilus californianus,* with special reference to the latitudinal distribution of the species and under weight controlled conditions. Though it is possible that the latitudinal differences in a species might be due to several factors, such as local nutritional conditions, oxygen and mineral content of the waters, for the present study temperature differences only are experimentally analyzed and correlated with the temperatures of the localities of origin of the animals.

It is a pleasure to acknowledge my debt to Dr. Theodore H. Bullock, not only for providing me with all the necessary facilities, but also for his enlightened interest in

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every aspect of the investigation. To Dr. John L. Roberts for several courtesies extended to me, I am greatly indebted. Thanks are due to other members of the staff of the Zoology Department, for their generous attention to my several needs. Particular mention must be made of the kindness with which Dr. Emery F. Swan sent me mussels and detailed data from Friday Harbor, Washington.

Material and Methods

Mytilus californianus Conrad, collected from Los Angeles (Lat. 34° 00′ N), Fort Ross (Lat. 38° 31′ N) and Friday Harbor (48° 27′ N), were used in the present investigation.[2] The mussels from the north were transported in moist air in insulated boxes and in both cases were transferred to aquaria of fresh sea water at a temperature chosen to approximate that at which they were collected not later than 48 hours after removal from their natural waters. To correspond with this treatment, local mussels were kept in insulated boxes after collection, for similar lengths of time, before being transferred to aquaria. Throughout the experimental period the mussels were kept at the supposedly natural temperatures which were 16°, 10° and 6.5° C., all ± 1.5° C., for Los Angeles, Fort Ross and Friday Harbor respectively. Sea water in the aquaria was continually aerated but was not running; it was changed once a week. This effectively eliminated the factor of excessive reduction in the salinity of sea water due to condensation at low temperatures. Over a week the salinity of the sea water in aquaria at 6.5° C. fell from 34.3% to 31.0% in extreme cases. The animals were not obtaining significant quantities of food.

The method described by Jørgensen (1943, 1949) was used for determination of the rate of water propulsion. This method depends on photoelectric estimation of the rate of clearing of suspensions of colloidal graphite. The same batch of graphite (Prodag, particle size 5–15 μ) was used throughout the course of this investigation, although it was observed that the values obtained by using Aquadag (particle size 1–2.5 μ) were not different. Pumping rate was not affected by the concentration of the suspension or its age within the limits used. Pumping rates as measured may not necessarily be equated with feeding rates, but the values obtained showed insignificant variation (see legend, Fig. 3) and correspond well with the values Jørgensen (1949) obtained on *Mytilus edulis* with flagellates and diatoms and which he interpreted as due to normal feeding with a "feeding mucus." The fact that the measured rates are constant for several individuals of any given weight from the same population, at any given temperature, permits of a comparative study. Each individual mussel was experimented upon from three to five times at each temperature, in vessels of 2 to 2.5 liters capacity. Ovigerous individuals were eliminated not only because they would effect the weight-specific rates but also because they were erratic in their activity.[3]

[2] All the mussels were collected from about 1½ to 2 feet above the 'O' tide level.

[3] Since writing the above account, data (to be published later) have been collected, which show that mussels from the low inter-tidal have a faster pumping rate compared to those 4 to 6 feet higher up on the same piling or rock surface. Further, a tidal-rhythmicity of the rate of water propulsion (which persists for over four weeks in the laboratory) in *Mytilus californianus* has been discovered. These two factors account for some of the variation reported above.

Results

Shell-weight as a function of latitude

While readings of weight of soft parts were being taken, it was noticed that the mussels from northern latitudes had relatively smaller percentage of the total weight as soft parts. Within the weight range recorded, shell weight is directly proportional to the wet weight of soft parts at any given latitude (Fig. 1A). The slopes of the lines show a consistent increase with latitude. When the shell weight is expressed as a percentage of total weight and is plotted against latitude (Fig. 1B),

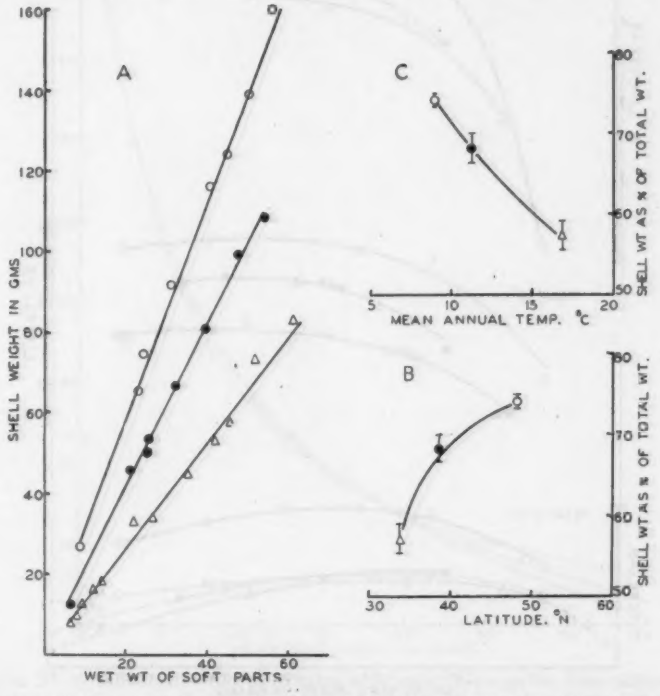

FIGURE 1. Weight of shell as a function of latitude in *Mytilus californianus*. △ Los Angeles; ● Fort Ross; ○ Friday Harbor. Mean annual temperature data from Scripps Institution of Oceanography, La Jolla.

there is an initial rapid increase in the percentage of shell weight, which flattens toward the higher latitudes. But, if the same percentage values of shell weight are plotted against mean annual temperature of the given locality (Fig. 1C), there is a close relation between decrease in temperature and increase in percentage of shell weight. Factors like duration of exposure, crowding and nature of the surface might influence this ratio at local areas. Thus Fox and Coe (1943) obtained a

higher percentage of shell weight for mussels cultured under water without exposure, at La Jolla. These results are exceptions to the common generalization that animals of cold northern latitudes tend to have thinner and more fragile shells (Wimpenny, 1941).

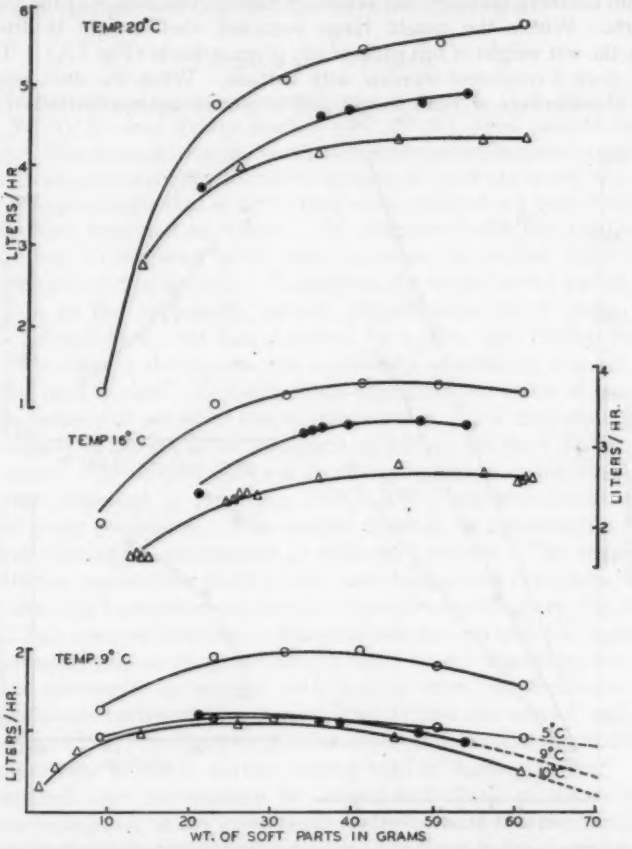

FIGURE 2. Absolute rate of pumping as a function of weight at different temperatures in *Mytilus californianus* from different latitudes. △ Los Angeles; ● Fort Ross; ○ Friday Harbor. Each point represents the average for five to twenty-five readings. Each weight group consisted of three to five mussels. See Figure 3 for statement of variation.

Rate of water propulsion as a function of latitude

A comparison of the effect of temperature on the absolute rate of pumping in animals of increasing weight, from different latitudes (Fig. 2), indicates that at any given temperature animals of equal weight from higher latitudes have a higher total pumping rate than those from more southerly latitudes.

At minimal temperatures (bottom, three curves) the absolute rate of pumping increases with increasing weight, until a certain point and then declines with increasing weight. This decline in absolute pumping rate with increasing weight is slower in higher latitudes. The fact that at their local minimal temperatures the northern animals can grow larger and yet remain as active as a much smaller animal

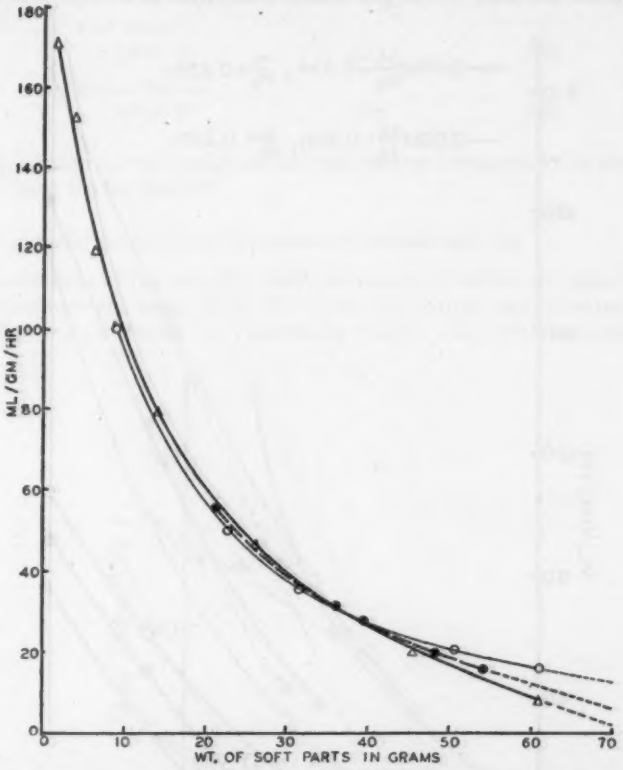

FIGURE 3. Weight-specific rate of pumping of *Mytilus californianus* from different latitudes at approximately minimal temperatures at these latitudes. △ Los Angeles at 10° C.; ● Fort Ross at 9° C.; ○ Friday Harbor at 5° C. Variation of rate in either direction not greater than $\frac{1}{2}\sqrt{n}$, where n is ml./gm./hr.

from a southern locality at its own minimal temperature, correlates well with the known larger size of animals from higher latitudes.

The weight-specific rate of pumping of the mussels from different latitudes appear identical at the minimal temperatures encountered at the respective latitudes (Fig. 3) (according to best available sources—Scripps Institute of Oceanography and U. S. Coast and Geodetic Survey), except that the decline in rate of activity per gram in larger animals is slower in samples from the higher latitudes.

In the cold-adapted, higher latitude animals, the smaller individuals are the first to be affected adversely by high temperatures (Fig. 2, top curve).

Comparing the rate of pumping per unit weight in *Mytilus californianus* of similar weights from different latitudes (Fig. 4), it is evident that at any given temperature animals from the higher latitudes have a much higher rate per unit weight than those from lower latitudes. A 20 gm. animal from either Los Angeles or Fort Ross

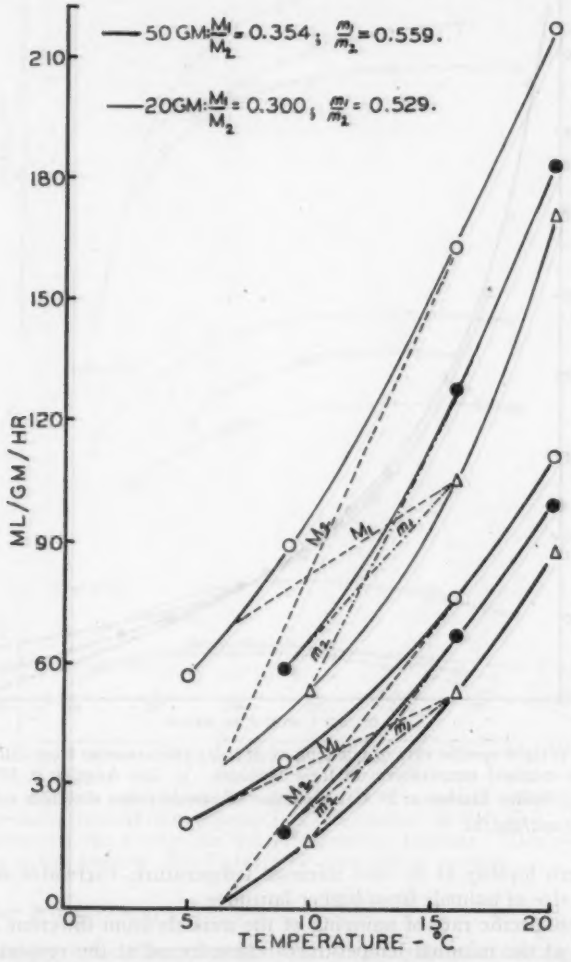

FIGURE 4. Rate of water propulsion as a function of latitude in *Mytilus californianus*. △ Los Angeles; ● Fort Ross; ○ Friday Harbor. Variation of rate in either direction not greater than $\frac{1}{2}\sqrt{n}$, where n is ml./gm./hr.

TABLE I

Q_{10} of rate of water propulsion of Mytilus californianus as a function of latitude and size

Latitude	Q_{10}(10–20° C.) 20 gm.	50 gm.
Los Angeles: 34°00′ N	3.2	5.44
Fort Ross: 38°31′ N	2.71	3.96
Friday Harbor: 48°27′ N	2.36	3.08

shows a higher rate per unit weight at all temperatures compared to an animal weighing 50 gm. from Friday Harbor.

Q_{10} of rate of water propulsion as a function of latitude and size

An examination of the attached table, the results of which are plotted in Figure 5, reveals that over the range of 10–20° C. the Q_{10} for the rate of water propulsion in *M. californianus* increases with increasing weight, while with increasing latitude

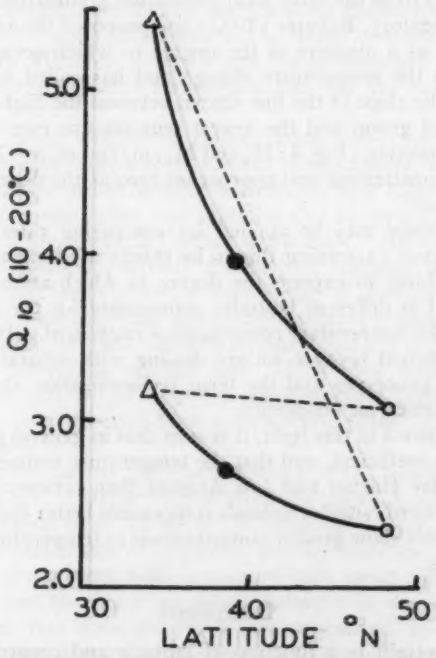

FIGURE 5. Q_{10} as a function of latitude in two different weight groups of *Mytilus californianus*. Bottom curve, 20 gm. individuals; top curve, 50 gm. individuals.

it decreases. Roberts (1952) similarly shows that the Q_{10} for oxygen consumption in *Pachygrapsus crassipes* increases with weight. The change in Q_{10} over the whole range of temperature for each group indicates that the phenomenon reported here is real and is physiologic. Hitherto, it has generally been assumed, chiefly on the basis of the data of Edwards (1946) on insects, that the smaller individuals show greater response to temperature change. A closer examination and analysis of the same data reveal that in *Melanotus* the larger individuals have a slightly higher Q_{10} at all temperatures. Especially marked is the difference at the lower temperatures recorded. The small individuals are not more sensitive to temperature change but have a higher rate at any given temperature, just as in the present case.

This relation between weight and Q_{10} is obviously of the greatest consequence in comparing different weight groups and in the ecology and distribution of poikilotherms.

Temperature compensation coefficient

A comparison of the rates of pumping of mussels of the same weight from different latitudes reveals that *M. californianus* from Friday Harbor has the same rate of pumping at 6.5° C. as the one from Fort Ross at 10° C., while animals from Los Angeles show the same rate at about 12° C. In comparing the degree to which groups of individuals from the same local population acclimatize to high or low temperatures in the laboratory, Roberts (1952) has proposed the ratio between the two slopes, M_1 and M_2, as a measure of the degree to which acclimatization has been achieved relative to the temperature change and has called it the acclimatization coefficient. M_1 is the slope of the line drawn between the highest temperature rate in the warm-adapted group and the lowest temperature rate in the cold-adapted, while M_2 is just the reverse (Fig. 4: M_1 and M_2; m_1 and m_2). This ratio is less than 1 if there is any acclimatization and approaches zero as the degree of acclimatization approaches perfection.

The same procedure may be applied for comparing rates of activity relative to latitudinal difference (assuming this to be chiefly a temperature difference), or, which is the same thing, to express the degree to which animals belonging to the same species located at different latitudes compensate for the temperature change. This may be called the *temperature compensation coefficient.* It cannot be called the acclimatization coefficient because we are dealing with separate populations which may have different genotypes and the term acclimatization should perhaps be reserved for purely phenotypic adaptation.

Interpreting Figure 4 in this light, it is seen that in general smaller animals have lower values for the coefficient, and that the temperature compensation coefficient is lower between Friday Harbor and Los Angeles than between Fort Ross and Los Angeles. In other words smaller animals compensate better than larger animals and more northern mussels show greater compensation to temperature change than those from lower latitudes.

Discussion

That the shell weight is a function of latitude and consequently of the mean annual temperature, is of interest, inasmuch as this morphological feature can be used to determine the physiological nature of a population. The consistency and

small variance of the shell weight also permit its use as an indicator of the temperature of the environment. The relation with the environment may be as close as for the familiar "rules" for vertebrate coloration and proportions.

It is shown that the absolute rate of water propulsion (ml./hr./individual) at minimal habitat temperatures begins to fall after a certain weight is reached, and that this decline is slower in higher latitudes than in lower. The weight-specific rate (ml./hr./gm.), of course, falls with growth and the correlation between the larger size of the northern forms and their relatively slower decrease in activity per gram, plotted against size is similarly significant. Though this does not necessarily imply that the maximal weight to which animals can grow in a given locality can be computed with accuracy by an extrapolation of the available data, still it does show that small differences in temperature are of great significance and that a physiological basis can be found for a well known generalization in ecological geography. This phenomenon of smaller size-dependence of activity in higher latitudes emphasizes a need for obtaining geographically and temporally closely spaced temperature records in inshore waters. The work of Hubbs (1948, 1952) in this direction is noteworthy and is a timely beginning.

In this connection special interest is attached to populations of offshore, deeper water individuals of the same species, particularly of sessile forms like *Mytilus* which have widely dispersed gametes and larvae. If the physiological differences found in populations widely separated latitudinally are based on phenotypic acclimatization, it is to be expected that the same differences will be found between intertidal populations and those at 20 to 30 meters depth, where the temperature is several degrees lower. Adequate material for this comparison has not yet been studied but is available.

The importance of taking weight into consideration when comparing individuals or groups for their activity, especially at various temperatures, is shown not only by the fact that at all temperatures smaller individuals have higher rates as is well known, but by the more significant fact that the Q_{10} as well as the temperature compensation coefficient are lower in smaller individuals. An examination of the data of Fox *et al.* (1936, 1939), already referred to, suggests that almost all the cases in which, at a given temperature, a *higher* rate of activity is reported from *lower* latitudes, are the result of using significantly smaller animals from the lower latitudes. The latter were, in these particular cases, from 2.3 to 45 per cent of the weight of the higher latitudinal animals with which they were compared. Of course the same factor acts to increase the significance of those cases reported where the forms from lower latitude have a *lower* rate at any given temperature, as the authors recognized.

In Figure 5, it may be noticed that the difference in Q_{10} value between a small animal from the north and a larger animal from the south is much greater than that between a small animal from the lower latitudes and a larger animal from the higher latitudes. This means that an organism moving from the northern latitudes toward the lower as it grows must make a tremendously great physiological adjustment as compared to one moving from lower latitudes to the higher. Further, it was observed earlier that amongst the higher latitudinal animals, individuals which are smallest are the first to be affected adversely by raising temperatures. These two facts make it probable that the center of distribution in a species like *Mytilus californianus* lies in the lower latitudes.

The use of the temperature compensation coefficient as a quantitative measure of regulation makes possible analysis and comparison of otherwise not easily comparable physiological phenomena. Shifting an animal from its normal temperatures evokes, in organisms like the mussel, a compensatory response. The degree to which an organism responds and compensates may be related to the degree to which it is removed from its norm. As observed earlier, mussels from more northern latitudes show greater compensation to temperature change than those from intermediate latitudes, not only absolutely but relatively to the temperature difference. Instances which may be parallel are found in human metabolism. Thus, the net efficiency of excess metabolism (over the basal), while walking on level is 20 per cent whereas the net efficiency of excess metabolism, while walking on an 11.4 per cent grade, is 52 per cent (Bazett, 1949). Rothstein and Adolph (1944) found that the amount of voluntary dehydration in acclimatized men is higher, the higher the rate of sweating. Under conditions causing men to sweat less than 400 gms./hr., 95 per cent of the water lost was replaced, whereas men working on the desert and sweating 1000 gms./hr. replaced only 50 per cent of their loss. Gross (1952) reports that regulation against osmotic stress in crabs is greater the greater the stress. The same seems to be true in the regulation of basal metabolism by precise control of heat loss in arctic as compared with tropical mammals and birds (Scholander, *et al.*, 1950). Out of this seems to evolve a principle: the relative compensation for environmental stress increases with increasing departure from a norm, within limits.

Summary

1. Shell weight relative to the weight of soft parts is a constant in any given population of *Mytilus californianus*, but it increases with increasing latitude.

2. The absolute rate as well as weight-specific rate of pumping is greater at any temperature in mussels from higher latitudes than in those of the same weight from lower latitudes.

3. The rate of decline, in the absolute as well as weight-specific rate of pumping, with increasing size, is shown to be slower in higher latitudes than in the lower. This perhaps affords a physiological basis for the larger size of the more northern forms.

4. The Q_{10} of the rate of pumping, between 10 and 20° C., is higher in larger individuals of any given population. But the Q_{10} of individuals of the same weight at different latitudes, shows a decrease with increasing latitude.

5. It is suggested that the center of dispersal of species like *Mytilus californianus* is in the lower latitudes. This suggestion stems from the fact that the difference in Q_{10} values between smaller individuals of lower latitudes and larger individuals of higher latitudes is negligibly small, while the opposite comparison results in a large difference, requiring a great physiological adjustment.

6. A comparison of relative degrees of compensation to temperature at different latitudes is made possible by a proposed temperature compensation coefficient. If the phenomenon under study is a phenotypic change in the lifetime of individuals, this becomes a measure of the degree of acclimatization (Roberts, 1952). This coefficient shows a greater degree of compensation for temperature in more northern samples and in smaller individuals.

7. Such a comparison, and several other instances from earlier work, seem to suggest that the relative compensation to change increases with increasing change.

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REPRODUCTION IN PHORONOPSIS VIRIDIS. THE ANNUAL CYCLE IN THE GONADS, MATURATION AND FERTILIZATION OF THE OVUM

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The question of the development, maturation and fertilization of the gametes is one of the many aspects of the biology of the Phoronidea on which available information is very incomplete. There are two accounts of gonad development (Ikeda, 1903; de Selys Longchamps, 1907), but there have been no previous studies of the maturation and fertilization of the egg. A related problem concerns the possibility of protandry in species other than the three known to be hermaphrodite. In most cases studies have not been sufficiently detailed to allow for a decision between protandry and dioecism.

The following account is concerned with an apparently dioecious phoronid, *Phoronopsis viridis*. It deals with the cyclic changes in the gonads, the maturation and fertilization of the egg, and includes some observations, made incidental to the main body of work, which bear upon the question of protandry in this group.

Methods

Phoronopsis viridis was collected from a tidal mud flat, Elkhorn Slough, situated in Monterey County, California, for a period of over 12 months at intervals of approximately four weeks. Before examination individuals were removed from their tubes by breaking the latter with fine forceps. Specimens from each collection were preserved in Bouin's fixative. Individuals used in the study of the gonads and nephridia were sectioned using either the paraffin or freezing technique. Paraffin sections were stained with Harris' hematoxylin and counterstained with alcoholic eosin. Material to be cut as frozen sections was bulk stained in alum cochineal before embedding in gelatin.

Eggs were obtained from mature specimens of *Ph. viridis* by puncturing the body wall in the reproductive region and allowing the ova to fall out. Since these eggs were found to be already fertilized they were simply pipetted into bowls of fresh sea water and allowed to develop at approximately 13° C.

Ova to be sectioned were fixed in Carnoy's fluid, stained whole by the Feulgen technique and counterstained in fast green. They were placed in a drop of horse serum on a slide, and the slide supported above a 50:50 mixture of glacial acetic acid and full-strength formalin in a warm petri dish. Within a few minutes the horse serum coagulated, and a small block containing the ova could then be cut from the drop on the slide. This block was then dehydrated, cleared, using tertiary butyl

[1] This work was done while the author was a graduate student in the Department of Zoology at the University of California, Berkeley. The author would like to thank Dr. W. E. Berg, Dr. R. I. Smith and Dr. R. M. Eakin of that department for valuable encouragement and criticism during the progress of the work.

alcohol, and imbedded in paraffin. Sections were cut at 5 μ and drawings of the sections were made with the aid of a camera lucida.

Whole mounts of ova stained in phosphotungstic acid hemotoxylin or by the Feulgen technique were also studied.

General Structure

Phoronopsis viridis was described by Hilton (1930) from specimens found at Moro Bay in southern California. The animals from Elkhorn Slough fit Hilton's description well with regard to those characteristics customarily used in the taxonomy of this group. These features are the general appearance, size, degree of spirality of the lophophore, number of tentacles and of longitudinal muscles and the position of the longitudinal nerve cord.

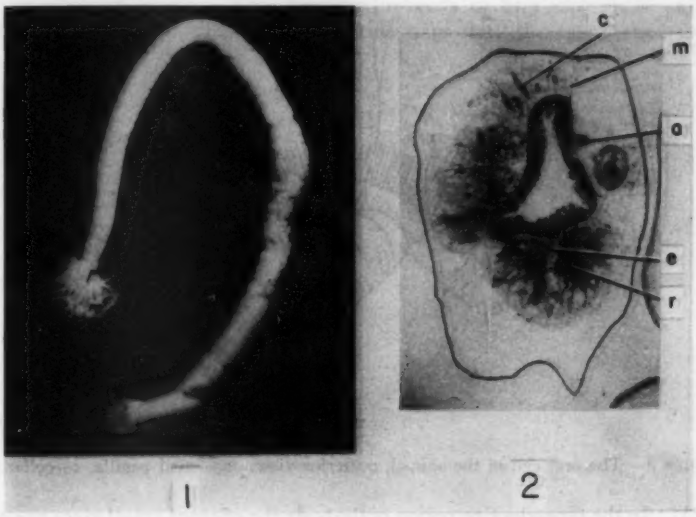

Figure 1. *Phoronopsis viridis.* The animal removed from the tube.

Figure 2. A cross-section of the body taken through the reproductive region. The reproductive tissue may be seen in the two anterior coelomic chambers. a—afferent vessel, c—capillary caecum, e—efferent vessel, m—mesentery, r—reproductive tissue.

Phoronopsis viridis is found in intertidal flats where the substratum is quite firm, consisting of a mixture of mud and sand. The animals inhabit tubes of a transparent secretion to which are cemented sand grains. The tubes, which are straight and occur vertically, vary in length from about 4 to 6 cm. and in diameter from 2 to 3.5 mm. The lower end tapers sharply in the last 3 or 4 mm. and is closed. The upper end of the tube is open and is usually flush with the surface of the mud. When the mud flat is covered with water the tentacles may project from the open end of the tube. The body is quite extensible and may extend 2 or 3 cm. beyond the end of the tube when the animal is not disturbed. The tube fits the animal tightly and it

is difficult to remove the latter intact. The general appearance of the animal out of its tube is shown in Figure 1.

Both mouth and anus open at the base of the coiled lophophore which bears the tentacles. The anus is borne on a papilla which is situated between the two arms of the lophophore, and the paired nephridia open on either side of this papilla near its base (Fig. 3). Below the lophophore there is a fold in the body wall forming the collar, and below this again is the tubular body which contains the digestive tract, the longitudinal blood vessels and the gonad. There are four longitudinal mesenteries which divide this part of the body cavity into four coelomic spaces. As can be seen from Figure 2 these mesenteries are not evenly spaced along the circumference of the body and consequently the left and right posterior chambers are considerably smaller than are the two anterior chambers. The afferent blood vessel lies within the right posterior chamber and the efferent vessel within the left anterior chamber

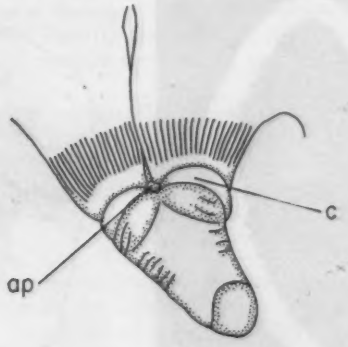

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FIGURE 3. The oral end of the animal, posterior view. ap—anal papilla, c—collar.

(Fig. 2). In the reproductive region of the body the efferent vessel gives rise to many short, blind branches, or capillary caeca (Fig. 2), and it is around these vessels that the gonads develop. The reproductive tissue is found only in the two anterior coelomic chambers, these areas being served by capillary caeca from the efferent vessel. The gonad is found in the aboral end of the body and may occupy from one- to two-thirds of the total length of the animal (Fig. 1). The body wall in this region is very thin and in season may become distended with gametes. The sexes are separate and may be easily distinguished during the breeding season, as the male gonad appears white and the female pink.

At times other than the breeding season a tissue known as the fat-body ("Fettkorper," Kowalevsky, 1867) or as vasoperitoneal tissue ("Vasoperitonealgewebe," Cori, 1939) is present around the capillary caeca. In life this tissue is jelly-like in consistency and transparent. Its structure, various inclusions and cycle of development are described below.

Annual Cycle in the Gonads

The developmental sequence in the gonad of *Phoronopsis viridis* is very similar to that described for *Phoronis ijimai* and *Phoronis australis* by Ikeda (1903) and for *Phoronis psammophila* by de Selys Longchamps (1907).

Female Gonad. The breeding season for *Ph. viridis* lasts throughout most of March and April. In the case of the female, the gonad in late February contains large numbers of full-sized ova, each about 60 μ in diameter. At this time most of the ova still possess the large germinal vesicle. They may be packed very closely and each is covered by a thin, squamous epithelium, one cell thick. The remnants of the vasoperitoneal tissue take the form of small patches or strands of granular cytoplasm between the masses of ova.

By about the middle of March many of the ova have become freed of the investing membrane. As this happens the germinal vesicle breaks down and the first maturation division begins. This division proceeds to first metaphase and stops (Fig. 4). The female nucleus remains at this stage until the egg is shed from the

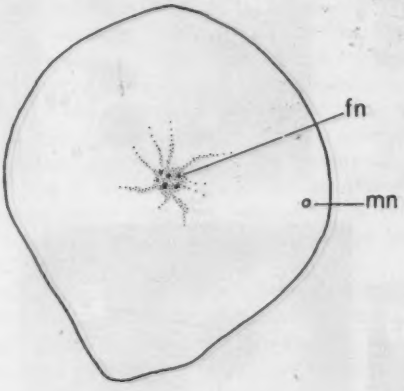

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Figure 4. Camera lucida drawing of an ovum from the body cavity. The female nucleus is in metaphase. From a female collected in March. fn—female nucleus, mn—male nucleus.

body. Once the ovum is freed of its investing epithelium it moves toward the periphery of the body and in the space between the outer surface of the gonad and the body wall the ripe ova collect. Occasionally an ovum will proceed to divide while still in the body cavity. Up to three or four such embryos have been found in one adult. Those found have always been blastulae. Similar embryos have been seen by de Selys Longchamps (1907) in *Phoronis mulleri* and *Phoronis sabatieri.*

Throughout the months of March and April large numbers of ova ripen and become free in the body cavity, where fertilization takes place. By the end of April most of the large ova have been spawned and there remain along some of the

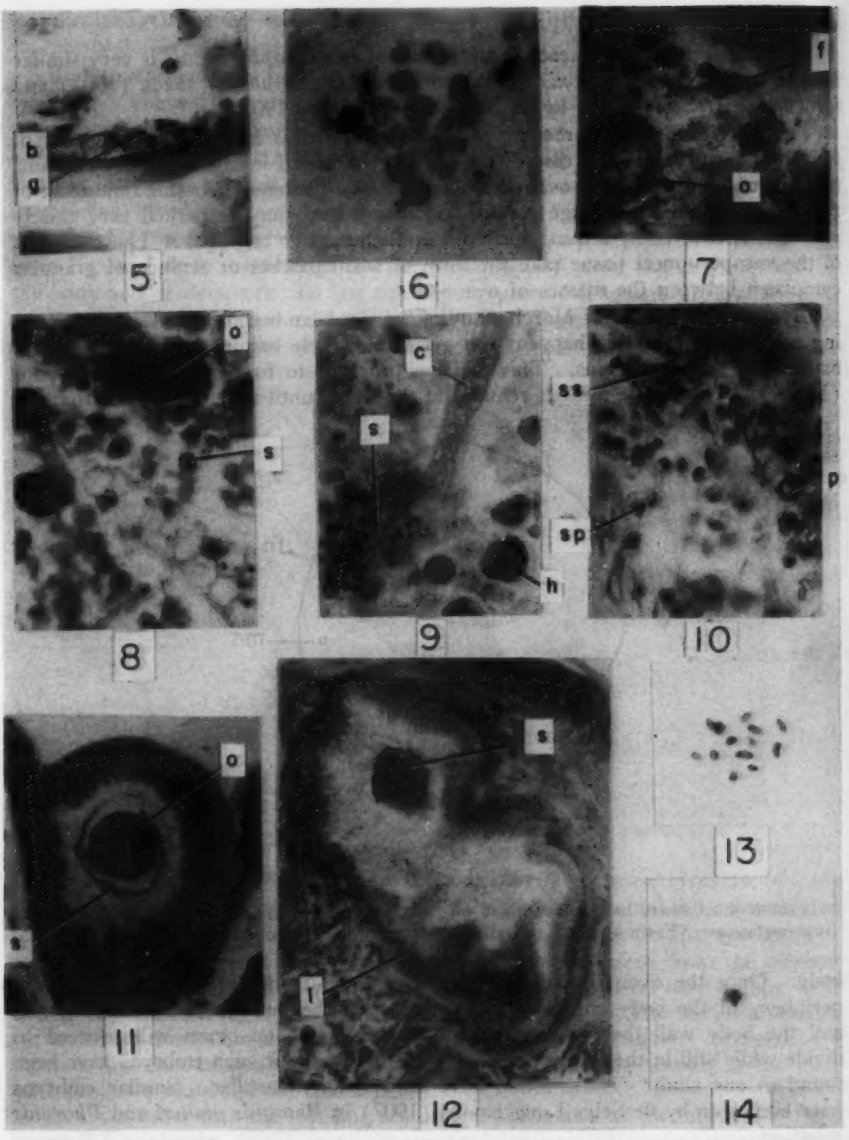

FIGURE 5. Opaque, non-staining granules, seen in a blood vessel. b—blood corpuscle, g—granule.

FIGURE 6. Dark green bodies, photographed from a smear of living tissue.

capillary caeca a considerable number of small ova, usually no more than 10 or 15 μ in diameter and frequently much smaller. These ova are presumed to be the forerunnners of next year's crop of eggs which began their development before this year's crop was shed.

At the end of April it is possible to examine some of the capillaries while they are entirely free of reproductive tissue. The walls of such vessels have been found to consist of a single layer of small, flat cells. These cells are said to be a part of the mesodermal splanchnic epithelium (Cori, 1890) and the capillaries, therefore, to be merely spaces between sheets of splanchnic peritoneum. Radiating from the capillaries are strands of tissue containing occasional nuclei, separating clear unstained spaces which are presumably filled with fluid in life and represent the spaces left by the discharge of the ripe ova.

In the body cavity of *Ph. viridis* and particularly in the reproductive region, there are a number of kinds of cells or bodies which may be found free in the coelomic fluid, or contained within the vasoperitoneal tissue. Such elements are present only in small numbers in and around the gonad at the breeding season, but may be found in this region in greater abundance at other times of the year. They have not been studied in sufficient detail to make possible an opinion of their functional role. There are, however, three types of bodies which have been seen repeatedly in almost all animals studied and these are described below.

The most obvious and frequently the most abundant of these elements is a fusiform body about 15 μ long. It stains a uniform pink with eosin, contains no apparent nucleus, and the surface is often marked with faint longitudinal lines (Fig. 7). Similar bodies have attracted the attention of many persons working with the Phoronidea and it has been suggested by Pixel (1912) that they are granules of nitrogenous waste material because of a positive reaction to the murexide test. As far as I know no other attempt has been made to determine their chemical nature.

In some, but not all of the animals studied there appeared groups of small, opaque granules (Fig. 5), which do not stain with either hematoxylin or eosin and which retain their color throughout fixation and dehydration. Their color under transmitted light is gold; they vary in size from 2 to 8 μ and have been found in the

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FIGURE 7. Fusiform, eosinophilic body, seen in a female animal. f—fusiform body, o—ovum.

FIGURE 8. Non-granular, eosinophilic spheres in the vasoperitoneal tissue. o—ovum, s—cosinophilic sphere.

FIGURE 9. Coarsely granular, hematophilic body in the vasoperitoneal tissue. c—capillary caecum, h—hematophilic body, s—spermatocytes.

FIGURE 10. Section through the gonad of a male animal collected in February. p—primary spermatocyte, sp—spermatozoa, ss—secondary spermatocyte.

FIGURE 11. Section through the nephridial duct of a female animal collected in March. o—ovum, s—spermatozoa.

FIGURE 12. Section through the nephridial duct of a male animal collected in March. l—columnar lining of duct, s—spermatozoa.

FIGURE 13. Aceto-carmine squash preparation of the metaphase nucleus of an ovum in the body cavity. There are 13 pairs of chromosomes.

FIGURE 14. Aceto-carmine squash preparation of the male nucleus found in an ovum in the body cavity.

blood vessels as well as in the body cavity. Similar granules have been described from *Phoronis psammophila* by Cori (1939), who believed them to be inactive and degenerate amoebocytes.

At any time of the year some individuals contain minute dark green bodies in the coelomic cavity (Fig. 6). These bodies are non-motile, occur singly or in groups of two or more, and may perhaps be algal cells.

Following spawning, the development of next year's ova continues and many new ova appear. The development of new ova is most rapid at the proximal ends of the capillary vessels, on the efferent vessel and on the walls of the blood sinus surrounding the digestive tract. The germ cells are at first small, roughly spherical and contain a few darkly staining granules. They are separated from the lumina of the capillaries by a single layer of flat cells and are covered by a similar layer. In the case of the lateral vessel these epithelial cells lie outside the muscular elements in the wall of the vessel. The reproductive cells appear to be produced continuously, so that any given length of capillary wall will bear a series of ova of different ages, the oldest lying farthest from the parent vessel.

As the new generation of ova is beginning to develop, the vasoperitoneal tissue reappears. As early as May the clear spaces left by the discharged ova begin to fill with a finely granular cytoplasm containing many eosinophilic spheres of various sizes (Fig. 8). The cytoplasm and granules appear to be contained within very large cells separated from one another by membranous walls containing the nuclei. These cells appear to vary in length from about 25 μ to 75 μ and in diameter from about 6 μ to 14 μ. In general they are arranged around the blood vessels so that the long axes of the cells are at right angles to and radiate from the long axis of the capillary. There may be exceptions to this pattern, but since the capillary caeca pass in a number of directions it is difficult to determine by means of sections the relationships of all the cells of the vasoperitoneal tissue. The size and arrangement of these cells is similar to that described for *Phoronis psammophila* by Cori (1939). The developing ova become imbedded in the extensive peritoneal tissue and push into it as they increase in size.

There are two main types of inclusions found only in the cells of the vasoperitoneal tissue. The more abundant of these is the above-mentioned non-granular sphere, varying in diameter from less than 1 μ to about 8 μ (Fig. 8). These spheres stain with eosin and come to fill most of the substance of the vasoperitoneal tissue. The other type of inclusion is a coarsely granular, roughly spherical body varying in diameter from about 5 to 15 μ. This type of body stains with hematoxylin and appears in greatest abundance in the late summer and fall (Fig. 9).

In July all the ova are small and are found chiefly around the proximal ends of the capillaries, close to the digestive tract. By November the reproductive tissue has spread along the blood vessels, covering them for most of their length. At this time the larger ova are about 35 μ long and many new ones are being formed. By January the ova have increased in size, the larger ones being about 50 μ long and usually somewhat flask-shaped. Each ovum is still surrounded by a single layer of flattened cells. The germinal vesicle is large and contains a diffuse chromatin network as well as a large nucleolus in the shape of a curved disc. In February the gonad is packed with many large ova, 50 to 60 μ in diameter, and there are still many small ones along some of the capillaries. By this time the vasoperitoneal

tissue has been almost eliminated; the inclusions found in this tissue in late summer and fall have disappeared and all that remains in the occasional spaces between ova are thin strands of tissue which probably represent the membranes of exhausted cells. It has been impossible to determine whether the developing ova penetrate into or between the cells of the vasoperitoneal tissue. Within the next month the ova ripen, are spawned, and the cycle of development of the gonads begins again.

Male gonad. Fully formed, active spermatozoa are found in male animals as early as December. The quantity of active male gametes increases throughout January, February and March. In March the aboral ends of male animals are usually a dense white due to the masses of spermatozoa inside. Spawning may take place in February, March and April.

In May, immediately after the end of the breeding season, there is apparently no reproductive tissue left. In contrast to the situation in the female, the tissue which in the male will give rise to next year's gametes does not appear until the breeding season is over and the present crop of spermatozoa shed. Later in May the vasoperitoneal tissue re-forms and the two types of inclusion described in the case of the female appear. At this time a few cells which are probably spermatocytes may be seen along some of the capillaries. By June the vasoperitoneal tissue is well formed and reproductive cells are abundant along the blood caeca. By August the quantity of reproductive tissue has increased considerably and throughout the fall months the proportion of generative tissue increases steadily as the extent of the vasoperitoneal tissue decreases.

The germ cells of the male arise in the walls of the blood vessels in the same manner as do those of the female. Spermatocytes can be distinguished readily in sections, but spermatogonia have never been satisfactorily identified. It seems probable that they resemble the primary spermatocytes. The latter are roughly spherical cells, about 3 μ in diameter, in which the nuclear material occupies most of the cell body. They are found immediately adjacent to the capillary walls, sometimes forming masses up to 20 cells deep. In some cases a single row of such cells along a capillary wall has been observed to be covered by a sheet of thin epithelial cells. No such epithelium has been seen in connection with larger masses of spermatocytes or their derivatives. The secondary spermatocytes are smaller, about 1 μ in diameter, and are typically found farther from the capillaries than the primary spermatocytes. The secondary spermatocyte consists almost entirely of nucleus and in some cases attempts were made to count the chromosomes. The highest number counted was 12. In December and throughout the breeding season the groups of spermatocytes become fringed with masses of fully formed spermatozoa. The latter appear first in pairs, often attached to one another at one end and for a part of their length (Fig. 10). Such pairs are usually distributed between the secondary spermatocytes and the single spermatozoa. During the breeding season clumps of spermatoza, aggregated so that the heads are together and the tails free, are frequently found in the body cavity of male individuals. When such clumps are placed in sea water they tend to disperse.

As in the female, the male gametes develop at the expense of the vasoperitoneal tissue. During the breeding season and immediately afterward the latter tissue is represented only by cytoplasmic strands radiating from the walls of the capillaries.

When spawning begins in the male there are many spermatocytes present, but by the end of the breeding season they have all disappeared and it is not clear whether they all develop to spermatozoa or whether some spermatocytes degenerate without reaching maturity. It is also possible that the small size of the spermatocytes may render them so inconspicuous in small numbers that under these conditions they appear to be absent. At any rate, a fairly careful study has failed to reveal any male reproductive tissue in the gonad immediately after the close of the breeding season.

It seems likely that both sexes spawn more than once during the breeding season. Sections of the gonad taken in March and April show areas depleted of gametes as well as areas filled with mature germ cells. The mid-body region of such animals is usually not filled with gametes and it seems probable that the eggs and spermatozoa from the depleted areas of the gonad have already been spawned. In the laboratory the animals usually spawn at night, emitting a slow stream of eggs or spermatozoa, but the quantity of material released under these conditions must certainly represent only a fraction of the total quantity produced.

Protandry

It has been suggested by a number of authors that some species of Phoronidea may be protandric, being first male and then female. It seems certain in the case of *Ph. viridis* that at any one breeding season an animal is either male or female. Moreover, from a study of the development of the female gonad it seems probable that a female at one breeding season will be a female at the next, as the ova for the following year are already present in small numbers. In the case of the male the evidence is inconclusive. The reproductive tissue disappears or else becomes exceedingly inconspicuous after spawning and once the season is over there is no way of recognizing a male except by the absence of ova.

If *Ph. viridis* were truly protandric, all individuals when they first reached sexual maturity would be males, and at some subsequent time would become females. If this were so it would be reasonable to expect that males might tend to be smaller than females. There is a considerable range of size among mature individuals of this species, but the length of life and rate of growth are unknown. Also it is well known that individuals may cast off the lophophore and tentacles when disturbed, and it is possible that constriction resulting in separation may occur in other parts of the body. Cases of fission of this type have been reported for *Phoronopsis albomaculata* (Gilchrist, 1907) and for *Phoronis ovalis* (Marcus, 1949). If this type of asexual reproduction does occur in *Ph. viridis*, then the length of the adult body may bear no direct relationship to the age of the animal. There has, however, been found no good evidence of asexual division in this species and hence measurements were made of the lengths of a number of male and female individuals to determine whether or not the sexes showed any significant difference in length.

These measurements were made throughout one breeding season (1949) on all animals removed intact from their tubes. A total of 169 animals was measured, of which 95 were males and 74 were females. The average length of males was 5.6 cm. and of females 6.75 cm. The standard deviation was 1.35 in the male group and 1.01 in the female group. The standard error of the difference between the means

of male and female populations (d/d) was calculated according to the formula in Simpson and Rose (1939), and was found to equal 0.545. When this value is referred to the tables of "t" the probability is found to be 0.6 (p value).

From this it is apparent that there is no significant difference in length between male and female individuals. The males are not significantly smaller than the females, as might be expected if the animals were protandric. This analysis does not, of course, prove that *Ph. viridis* is not protandric; it merely indicates that on the basis of length of body there is no evidence for protandry.

Spawning, Fertilization and Maturation of Ova

The gametes of *Ph. vididis* pass out of the body by way of the nephridia. There are two nephridia which open to the exterior on either side of the anal papilla. Each nephridium is roughly U-shaped; the longer arm of the U opens to the exterior by a small pore; the shorter arm terminates in a pair of long funnels. One funnel of each nephridium opens into the posterior coelom and one into the anterior coelom. The walls of the duct are lined with columnar epithelium bearing very long, fine cilia (Fig. 12). The cells lining the funnels are also columnar and ciliated, but are narrower and more densely packed. The nephridia extend through the collar region of the body and are about 0.5 mm. long.

Examination of living animals at the time of breeding reveals that the contents of the reproductive region of the body are in constant motion as a result of contractions of the muscles of the body wall in the mid-body and reproductive regions. It seems probable that these movements are instrumental in getting the ova, once freed of investing membranes, up into the oral end of the body near the funnels of the nephridia. The average body length is about 5 or 6 cm.; the ova mature some 3 cm. from the nephridial funnels and must in some manner be propelled this distance before spawning can take place.

Sections of male animals, preserved in March, at the height of the breeding season, show large numbers of spermatozoa in the nephridium (Fig. 12). The spermatozoa occur in concentrated, roughly spherical masses which are found in both arms of the nephridium.

Sections of female animals preserved in March show both ova and spermatozoa in the lumen of the nephridium and in the body cavity (Fig. 11). The spermatozoa in the female nephridium may occur in dense masses and in some cases an ovum may be seen passing through a loose mass of spermatozoa (Fig. 11). Similar masses have been found in the mid-body region of a female collected in January. During the months of February, March and April spermatozoa may be found dispersed throughout the reproductive region of the female. No male tissue has ever been found in an otherwise female animal and it is probable that the spermatozoa which appear in the body of the female in January or February have come from a male animal. It seems likely that the spermatozoa enter the body of the female through the nephridium and fertilize the ova as they become free in the coelom. A male nucleus can usually be seen in ova which are free in the coelom and in which the female nucleus is in first metaphase (Fig. 4), but has not been found in ova which are still covered by the investing epithelium and in which the germinal vesicle remains intact. The male nucleus is very small and has been identified only by means of the Feulgen technique.

Once the ova has passed, by natural or by artificial means, from the body cavity into sea water the egg nucleus becomes active. Aceto-carmine squash preparations of ova from the body cavity reveal 13 pairs of chromosomes (2 n complement) on the metaphase plate (Fig. 13). The male nucleus may be seen in the preparations, although the chromosomes are not distinguishable (Fig. 14). Ten minutes after release into sea water the chromosomes of the egg nucleus have passed from metaphase to anaphase. At fifteen minutes the first polar body is forming. Thirty minutes after liberation the chromosomes are in metaphase of the second maturation division which takes place near the surface of the egg, usually very close to the first polar body. As the second polar body is forming the first usually divides.

During the first thirty minutes after liberation, while the two maturation divisions are taking place, the male pronucleus remains inactive. It may be found in a variety

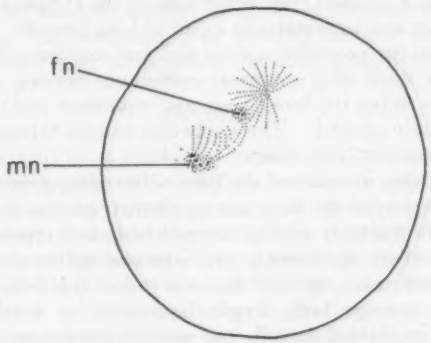

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FIGURE 15. Camera lucida drawing of an egg fixed forty minutes after liberation from the body cavity. Asters are forming around both male and female pronuclei prior to the formation of a fusion nucleus. fn—female nucleus, mn—male nucleus.

of positions within the egg, usually at some distance from the female pronucleus. Shortly after the formation of the second polar body the individual chromosomes of the male pronucleus become visible and an aster forms around them (Fig. 15). About forty-five minutes after liberation, both male and female pronuclei have moved to the center of the egg, forming a fusion nucleus of short duration. One hour after liberation this nucleus is in metaphase or anaphase of the first cleavage.

As the polar bodies are being formed it becomes apparent that the egg is invested in a thin, closely fitting membrane which is now detectable by virtue of being lifted from the surface by the extrusion of the polar bodies.

DISCUSSION

Gonads. From the preceding account it is clear that the yearly turnover in the gonads involves two cyclic processes in opposite phase. One of these is the cycle of development of the sex cells, beginning in the months of June and July and cul-

minating in mature gametes in the following March and April. The other cycle is that of the vasoperitoneal tissue which reaches its ebb at the time of the breeding season and its peak in July and August in the male, or in September and October in the female. The vasoperitoneal tissue thus reaches its greatest development at a time when the gonads are at their smallest, and as the gonad increases in size the vasoperitoneal tissue becomes commensurately reduced. This relationship, together with the intimate contact which exists between the developing gametes and the vasoperitoneal tissue, has led other investigators (Ikeda, 1903; de Selys Longchamps, 1907) to assume that the substance of the vasoperitoneal tissue nourishes the growing germ cells and is therefore exhausted at the time of the ripening of the latter. Ikeda (1903) considers that the cells of the vasoperitoneal tissue act as follicle cells in relation to the gametes, and that the latter absorb nutrient from the vasoperitoneal cells. De Selys Longchamps (1907), however, holds that the spermatocytes and oocytes become free in the body cavity, pass into the cells of the vasoperitoneal tissue and there complete their development. Observations on *Ph. viridis* show that as the ova develop they are covered by a layer of epithelium which disappears, releasing the ova into the body cavity at the time at which the germinal vesicle breaks down. In the case of the male there is no detectable investing membrane and it is possible that the spermatocytes do undergo at least a part of their development in the body cavity. In either case, however, there is no evidence that the germ cells penetrate into the vasoperitoneal cells. It certainly seems possible that the vasoperitoneal tissue nourishes the germ cells in *Ph. viridis*, but the exact nature of the relationship between the two tissues is not clear.

After spawning there remain strands of tissue radiating from the blood caeca and between these strands are the spaces left by the discharge of the ripe gametes. It has been assumed that these strands are the remains of the vasoperitoneal tissue and, in the case of the female, also of the epithelium that once covered the ova. These spaces may be considered either as the now vacant intercellular spaces or, less probably, as the enlarged interiors of vasoperitoneal cells. Later in the year the radiating strands are not prominent and the vacant spaces fill up with new vasoperitoneal tissue. The relationship between the old strands and spaces and the new tissue is uncertain, although it appears that the new tissue gradually forms around the remains of the old. The fully formed vasoperitoneal cells appear to be constructed rather like watery sacs containing globules of various sizes, the nucleus being near the cell membrane. Ikeda (1903) states that in *Ph. australis* and *Ph. ijimai* the vasoperitoneal tissue arises by a proliferation of the peritoneal layer forming the walls of the blood vessels. Cori (1939) says that the situation is similar in *Ph. hippocrepia*. In *Ph. viridis* it has not been possible to determine the origin of this tissue.

Concerning the origin of the reproductive cells, Ikeda's (1903) account for *Ph. ijimai* and *Ph. australis* is in agreement with the findings for *Ph. viridis*. Although oogonia and spermatogonia have only been tentatively identified in *Ph. viridis*, it seems quite certain that they must arise from the peritoneal covering of the blood vessels as described by Ikeda.

Protandry. Apart from the work on the three species which have been shown to be hermaphrodite (*Ph. hippocrepia, Ph. ijimai* and *Ph. australis*) there is very little information concerning the question of protandric hermaphroditism versus

dioecism in this group. De Selys Longchamps, working with *Phoronis psammophila*, found that most individuals contained either testis or ovary, but he obtained one specimen in which the testis surrounded a group of developing oöcytes. On the basis of this evidence *Ph. psammophila* has generally been considered as a protandric hermaphrodite. In several other cases species collected at only one time of the year have shown only ovaries or testes (*Phoronis vancouverensis* and *Phoronopsis harmeri*, Pixel 1912, and *Phoronis mulleri*, de Selys Longchamps 1902). Torrey (1901) found only testes in specimens of *Phoronis pacifica* collected in Humboldt Bay in June, whereas specimens from Puget Sound collected in another year (month not given) contained ova in the nephridia. He suggested that *Ph. pacifica* might be dioecious. Marcus (1949) reports that *Phoronis ovalis* from Brazil contains testes in May and oöcytes in July but it is not known whether or not this species is hermaphrodite.

Brooks and Cowles (1905) have made a more detailed analysis of the cyclic changes in the gonad in *Ph. architecta*. In this species there exists a situation somewhat similar to that described for *Ph. viridis*. *Ph. architecta* sheds its eggs freely into the water and at the breeding season individual animals contain either testes or ovary, but never both. Male animals possess a lophophoral organ and female animals do not. The males are mature from March to October and the females from May to October. Brooks and Cowles decided that this animal was probably protandric, that the males which were mature in March and April, before the onset of egg laying, had developed from eggs laid early in the previous year, that is, in May or June, and that these male individuals having spawned in March and April of their first year would become females by May. The males which matured in May and later were considered to have developed from eggs laid late in the previous breeding season which had not yet had time to pass through the male phase and become female. The lophophoral organs were found to contain spermatozoa and were considered to act as storage places while the animal changed from the male to the female state. Although spermatozoa were found in the body cavity of females, fertilization was considered to take place in the tentacular crown.

If this hypothesis be applied to *Ph. viridis* several difficulties arise. In the first place there is no lophophoral organ and therefore no storage place for the spermatozoa. In the second place the breeding season for *Ph. viridis* is much shorter; the eggs are shed over a period of only two months as compared with six in the case of *Ph. architecta*. This means that individuals of *Ph. viridis* conceived at the beginning of the egg-laying period could at most be only two instead of six months older than those developing from eggs laid at the end of this period, and such individuals are therefore less likely to have had time to pass through the male phase and become female. Finally it seems that the theory of Brooks and Cowles is, in the case of *Ph. viridis*, a rather roundabout way of explaining a phenomenon which could be a result of a situation in which there was no change of sex during the breeding season, but simply one in which the males matured earlier than the females which would ensure an abundance of spermatozoa when the ova became ripe. The fact that large masses of spermatozoa are found in the nephridia of males suggests that these spermatozoa are shed by the males, and the presence of similar masses in the nephridia and body cavity of females suggests that these masses are collected by the female. The method of this collection is an interesting problem

which might be partially elucidated by a careful study of the currents of the branchial crown and lophophore. It is suggested that the spermatozoa are discharged in compact masses by male animals, some of whom will be no more than a centimeter from the nearest female. The masses of spermatozoa could then be drawn into the tentacular crown of the female by the currents created by the cilia on the tentacles and in some manner passed into nephridia. It is unknown whether the spermatozoa pass into the nephridium by active swimming movements or are swept in by the cilia on the nephridium. The presence of large, compact masses of spermatozoa in the female suggests that they may have been passed inward passively. Considering the probably normal excretory function of the nephridium and the size and position of the nephridiopore, spermatozoan entry is a difficult matter to explain satisfactorily.

Even assuming that the above course of events does take place, there remains the possibility of a change of sex from one year to the next, so that animals which are males one season will be female the following one. If this were so it might also be possible that the spermatozoa seen in the body cavity of the female were left over from the previous male phase and had not entered from the outside. This possibility seems somewhat unlikely since it would necessitate the maintenance of active spermatozoa for a period of eight months, would result in self-fertilization, and would not explain the presence of spermatozoa in the nephridia of both males and females, or the apparent lack of spermatozoa in the reproductive region of females from April to December.

On the basis of present information, therefore, it seems most probable that in *Ph. viridis* the sexes are separate, at least during any one breeding season, and that spermatozoa leave the male and enter the female via the nephridia. Since the evidence is by no means complete, the possibility of protandry cannot be ruled out, but it is at present considered unlikely.

Fertilization. Although spermatozoa have been seen in the body cavity of the female by a number of authors (Brooks and Cowles, 1905; de Selys Longchamps, 1907), they have not been previously described from the nephridium of the female and it has been generally assumed that fertilization took place outside the body, in the tentacular crown. Torrey (1901) and Brooks and Cowles (1905) were certain that the spermatozoa did not penetrate the eggs in the body cavity. Considering the small size of the male nucleus, however, it seems probable that *Ph. viridis* is not unusual and that the presence of two nuclei in the eggs in the body cavity may in the future be demonstrated in other species of phoronids. The fact that all phoronids investigated in this respect are alike in having the first meiotic division of ripe body cavity eggs arrested at metaphase suggests that they may also resemble one another with respect to the site of fertilization. It is not certain when the spermatozoon actually enters the egg of *Ph. viridis*, although it seems very probable that this takes place at the time of the breakdown of the germinal vesicle.

Summary

1. The seasonal changes in the gonad of *Phoronopsis viridis* are described and are seen to involve the proliferation after spawning of vasoperitoneal tissue which is subsequently reduced and largely replaced by developing reproductive tissue.

2. No significant difference between the body lengths of male and female animals was found in the group of 169 animals measured. There is thus no evidence for protandry on the basis of body length.

3. Spermatozoa were found in the nephridia of both male and female animals and in the body cavity of female animals from January to May. Ripe eggs in the body cavity, in which the female nucleus is arrested at first metaphase, also contain a male nucleus. Eggs still covered by the investing epithelium and in which the germinal vesicle is still intact do not contain a male nucleus.

4. *Ph. viridis* is considered to be probably dioecious. Spermatozoa are believed to enter the female through the nephridium and fertilization appears to take place in the body cavity of the female, following the breakdown of the germinal vesicle. The egg when spawned is already fertilized and the maturation divisions proceed as soon as it is shed into sea water.

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THE X-IRRADIATION OF MARINE GAMETES.[1] A STUDY OF THE EFFECTS OF X-IRRADIATION AT DIFFERENT LEVELS ON THE GERM CELLS OF THE CLAM, SPISULA (FORMERLY MACTRA)

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This study was made for the following reasons:

1. To determine the radiosensitivity of the sperm and eggs of the clam and to compare it with the radiosensitivity of Arbacia gametes.
2. To determine whether cysteine or the new Bacq solution (B-mercapto-ethylamine) has any protective effect on the gametes and early development of marine forms.
3. To determine whether activation and syngamy could be separated by x-irradiation, and, if so,
4. To determine whether cross-fertilization with irradiated gametes and inter-generic parthenogenesis could be accomplished.

Materials and Method

The eggs and sperm of the mollusc, *Spisula solidissima,* were used in these studies.

The eggs of the clam Spisula seem to be produced almost continuously, and (according to Allen) are available except for a period of about 10 days in August (Allen, 1951). The shell is cracked along the two-year line and the ovaries are cut out and dissected into small pieces on cheese-cloth suspended in a beaker of filtered sea water. The eggs pass through the cheese-cloth leaving behind the ovarian tissue. The ovarian masses are gently agitated to liberate the eggs. About one hour later, the sea water is decanted off and fresh (filtered) sea water is added in sufficient quantity to bathe thoroughly the eggs. This process is repeated after the eggs have settled so that the eggs get at least two thorough washings. After this they can be fertilized, or can be set aside at room temperature for use within 6–8 hours, or can be placed in the refrigerator at 10 degrees C. for use during the next 24–30 hours. The fertilization after 24 hours is still 100%, but drops to about 40% in 48 hours. Eggs in refrigeration are brought to room temperature and changed to fresh sea water before use.

The sperm are obtained by cutting the testes into small pieces in a minimum of sea water in No. 2 Stender dishes. These so-called "dry sperm" may be kept in covered dishes for dilution and use during 24 hours, or, if placed in refrigeration at 10 degrees C. may be kept for 48 hours longer. Just before use in fertilization,

[1] This document is based on work performed under Contract AT-30-1-GEN-70 for the Atomic Energy Commission.

one drop of dry sperm is added to 40 cc. of sea water (No. 2 Stender-full) and the suspension made homogeneous with pipette stirring. Then one pipette-full of this suspension is added to a finger bowl of eggs to secure normal fertilization of all eggs. If the sperm are refrigerated, they are given about ten minutes in suspension to become thoroughly activated. In all experiments involving irradiated sperm similar dilutions were made up after irradiation and similar amounts of diluted spermatozoa were used.

The two presumably protective chemicals used were cysteine hydrochloride and another — SH solution obtained from Dr. Bacq of Bruges, known as 1573L. It is B-mercaptoethylamine. Dr. Bacq has demonstrated (1951) the protective value of this drug for mice, and claims that it is even more effective than is cysteine. The cysteine experiments have been carried on largely at the Argonne Laboratories by Patt and his co-workers (1949, 1950).

The solution obtained from Dr. Bacq is in ampoules of 10 cc. each representing 100 mg., and has been demonstrated by him to be non-toxic but protective to mice against x-irradiation when used at levels of three mg. per mouse of average weight of 30 grams (Bacq *et al.*, 1951). The undiluted solution has a pH of approximately 7.5. It was diluted in filtered sea water and adjusted to a pH of 8.0. The concentrations used were designated in milligrams per cent. It was found that 0.5 mg. % was lethal to Arbacia plutei (used as test material) and that 0.01 mg. % was non-toxic to gametes and early embryos. Twice this concentration, 0.02 mg. %, was toxic for Arbacia plutei over a 24-hour period.

Cysteine hydrochloride was obtained in the crystalline form and it was found that a 1% solution in sea water had a pH of 1.9. This solution was made up fresh for each experiment. It was adjusted with concentrated NaOH to a pH of approximately 8 which is in the range of normal sea water. X-irradiation to 18,900 r did not alter the pH of the cysteine solution. Cysteine in solution is more stable as an acid and unless kept free from air (oxygen) and at refrigeration temperatures, it develops a flocculent sediment due to degeneration to cystine. It is known that cysteine activates enzymes, combines with H_2O_2 (which is known to be formed in irradiation solutions) and rapidly oxidizes to the non-protective SS-cystine. It was found that a 0.5% solution caused a slight shrinking of clam eggs and a wrinkling of the surface, suggestive of hypertonicity. A 0.1% solution had no such effect and cleavage to 100% could be obtained. However, development was abnormal, so that a solution of 0.01% was used for these experiments.

In all fertilization experiments controls were set up at the beginning and at the end of each series so that the time factor of the experiment was not involved. Regulation finger bowls were used, each of which had been pre-tested for possible contamination. This was done by using sea water and living Arbacia embryos kept over a period of three days. Each bowl contained 100 cc. of filtered sea water, and all bowls were kept under the same conditions of light and temperature.

The x-ray facilities were those of the Marine Biological Laboratory, Woods Hole, Mass. The factors were 182 kv, 25 ma with two alternate parallel tubes having equivalent filtration of 0.2 mm. Cu and output of 6300 r/min., under conditions used in these experiments. When eggs or sperm were to be irradiated for more than four minutes, the plastic fly box in which they were placed was surrounded by ice cubes so that heat emanating from the filaments of the x-ray tubes

would not affect the material. The plastic containers had been previously tested against living embryos for toxicity.

Observation and Experimental Data

Spisula

Spermatozoa

The spermatozoa of this genus are more radioresistant than are the eggs. This was determined by studying the effect of x-irradiated sperm on cleavage and on early development when used with normal, unirradiated eggs. The results were compared with those obtained when normal sperm were used with x-irradiated eggs.

Fertilization of normal eggs was accomplished to approximately 100% with concentrated (dry) spermatozoa exposed to all levels of x-irradiation from 3,150 r to 264,600 r. One can say, therefore, that the mechanics of fertilization seem to be in no way altered by the x-irradiation of spermatozoa of this species at any level studied.

Fertilizing power in all vertebrates naturally presumes motility on the part of the spermatozoa. However, motility alone is no guarantee of successful fertilization. Motility was not stopped in concentrated sperm irradiated to the maximum level of 264,600 r and then diluted. If the highly exposed spermatozoa are kept concentrated and left at room temperature (23–25 degrees C.) for 24 hours, only about 10% of the eggs are fertilized, and very few of these develop into motile trochophores. An exposure of concentrated sperm to 189,000 r followed by 24 hours in the refrigerator at 10 degrees C., did not alter the motility or the fertilizing power of Spisula spermatozoa. Many of the trochophores produced from such sperm and normal eggs appeared to be quite normal. With further refrigeration to 48 hours there was complete loss of fertilizing power by the spermatozoa. Controls, under similar conditions, gave normal fertilization and normal trochophores.

Cleavage delay of normal eggs fertilized by irradiated sperm was slightly increased with increasing exposures to x-irradiation.

Table I represents the average of six sets of readings, each of which involved thousands of eggs. Experiments were performed at the laboratory temperature of 23–25 degrees C.

Table I points up two possibilities. (1) The interval between the time of fertilization and the time of first cleavage of 50% of the eggs is increased by x-irradiation of the spermatozoa alone, but the increase is not linear nor does it follow any ratio with respect to the increased irradiation. The delay is increased to a maximum at about 113,400 r and then the curve shows a plateau of effect or even a slight decrease with further irradiation. This suggests that while the activating factors of the spermatozoon are not altered (*i.e.*, fertilization is achieved in all), the genetic material of the sperm, the syngamic or even the mitotic equipment, is so damaged that the initial cleavage is altered. The maximum delay of 15 minutes represents approximately the normal interval between the first and the second cleavages, so that possibly the first cleavage is actually omitted at 163,800 r or above. To substantiate this thesis further it might be pointed out that after the plateau is reached, there is never any reduction of the cleavage delay to a level which might be compared with the controls. Haploid (parthenogenetic) embryos have been produced among

other genera, and by other means, which showed such cleavage delay and abnormalities (Fankhauser, 1945; Parmenter, 1933, 1940; Harvey, 1936, 1940; Tyler, 1941; Tyler and Bauer, 1937; Rostand, 1938; Porter, 1939; Kawamura, 1939; Tchou-Su and Chen-Chou-Hsi, 1940). It should be emphasized further that cleavage of 50% of the eggs includes all eggs fertilized, and some of these may have been fertilized by spermatozoa of varying radiation damage so that the time lapse represents an average of the functional activity of all damaged sperm. It is likely that in these eggs there is an internal maladjustment of normal chromosomal material from the egg with variously damaged chromosomal material from the x-irradiated sperm (assuming that no two sperm were necessarily damaged in the same way). In some eggs the partially damaged sperm genetic material may have little adverse effect while in others it may be completely incompatible with the normal egg complement and thereby either kill the zygote or cause developmental abnor-

TABLE I

Sperm condition	Time from fertilization to first cleavage for 50% of eggs	Range of times	Delay in minutes	Trochophore %
Sea water control	58″	56– 61	0	100
6,300 r	1′02″	59–1′04″	4	100
12,600 r	1′03″	01–1′05″	4	100
18,900 r	1′05″	1′03–1′06″	7	72
25,200 r	1′07″	1′05–1′08″	9	45
31,500 r	1 09″	1′05–1′14″	11	62
44,100 r	1′10″	1′08–1′13″	12	55
63,000 r	1′11″	1′07–1′16″	13	48
89,200 r	1′13″	1′07–1′17″	15	60
113,400 r	1′12″	1′06–1′17″	14	45
138,600 r	1′13″	1′07–1′20″	15	40
163,800 r	1′10″	1′04–1′15″	12	33
170,400 r	1′09″	1′05–1′14″	11	40
189,000 r	1′10″	1′08–1′11″	12	65
214,200 r	1′12″	1′06–1′16″	14	85
239,400 r	1′11″	1′06–1′15″	13	95
264,600 r	1′10″	1′06–1′15″	12	95

malities (O. Hertwig, 1910, 1911; P. Hertwig, 1911, 1916; Rugh, 1939a, 1939b). Further (2) if one studies the trochophores (early embryos) arising from these fertilized eggs one finds that the low 6,300 r level of exposure as well as the very high 264,600 r exposure each produces nearly as many motile, ciliated trochophores as did the control sperm. The low point of trochophore production and maximum incidence of abnormalities is at about 163,800 r, suggesting that this level of exposure of spermatozoa causes the maximum of developmental incompatibilities. The abnormal trochophores also exhibit a wide range of sizes. Fertilization incompatibilities are overcome by further irradiation of the spermatozoa (Rugh and Exner, 1940). It is true that the trochophores from highly irradiated sperm are not normal and that they do not develop beyond 30 hours, but they are structurally quite uniform and appear to be very much like those of the controls. This may be due to the probability that these are haploid embryos. This further substantiates the thesis

that the greater the irradiation of the sperm the less deleterious effect will the sperm genetic material have on the developmental process of the resulting zygote and embryo.

Since concentrated spermatozoa can be exposed to 264,600 r and, if properly diluted, can still fertilize 100% of the available eggs and since these in turn become ciliated and motile trochophores within 6 hours at laboratory temperatures, it seems to be difficult if not impossible to obliterate or alter the fertilizing mechanism of the spermatozoa by x-irradiation. It is presumably only the genetic (chromosomal) material that is damaged and this occurs at the lower levels of irradiation. This is not evident, however, until the developmental processes are brought into play.

All the above observations were based on the x-irradiation of dry (concentrated) spermatozoa. It was obviously in order similarly to irradiate sperm at

TABLE II

*Fertilization percentage of normal eggs and irradiated sperm**

Exposure	Dilutions of Sperm**		
	1/20	1/200	1/2000
Controls	100	100	100
6,300 r	95	95	90
9,450 r	95	95	80
12,600 r	95	80	50
15,750 r	95	75	30
18,900 r	95	60	30
22,050 r	90	45	20
25,200 r	95	33	0
28,350 r	90	5	0
31,500 r	95	1	0
Controls***	90	95	80

* Values are per cent cleavage in all eggs studied.

** The number of sperm for each batch of eggs could not be controlled accurately. Nevertheless, the same method of sampling was used throughout so that it can be assumed that the numbers of available sperm were rather constant.

*** Since the process of irradiation involved time, a second set of non-irradiated controls was used at the termination of the irradiation period. In this way the time factor above could be included.

various dilutions to compare the radiosensitivity. Here the situation is quite different.

Concentrated (dry) sperm may be used for many hours, particularly if they are kept in the refrigerator in a covered dish to prevent desiccation. The sperm dilution experiments, to the contrary, had to be conducted within a relatively short time since it is well known that mere dilution will activate spermatozoa which have a limited reserve of energy and consequently a limited life (Lillie, 1919). One cubic centimeter of fluid may be roughly considered as 20 drops. Therefore, one drop of dry sperm added to 10 cc. of sea water was considered to be a 1/200 dilution. Likewise, one drop of dry sperm added to 100 cc. of sea water would be a 1/2000 dilution. The minimum dilution of sperm used was 1/20 or one drop of dry sperm

to one cc. of sea water. The same volume of sperm suspension was used in all fertilization experiments. In all cases the sea water was filtered and used at the laboratory temperature immediately. After a preliminary run it was found that irradiation of spermatozoa to dilutions of 1/2000 or more could not tolerate exposures above 31,500 r. Therefore it was decided to use graded doses of exposure up to 31,500 r only.

The results of these experiments are shown in Table II.

The more dilute the spermatozoa the more radiosensitive, as measured by the fertilizing power of irradiated sperm and normal eggs. Further, the concentrated sperm at any level produced (qualitatively) more normal trochophores than did the diluted sperm, suggesting that even the genetic material may have been more adversely affected by irradiation in the diluted state.

Presuming that the genetic contributions of highly irradiated sperm might be of no significance (based on parthenogenetic stimulation of Spisula eggs by Spisula sperm irradiated to 264,600 r), it was conjectured that such sperm might be able to stimulate the eggs of the echinoderm, Arbacia, and cause parthenogenetic development. Interspecific crosses of this type have been accomplished among the amphibia (Rugh and Exner, 1940). This, however, could not be achieved in even a single Arbacia egg in many attempts with thousands of eggs, and concentrated and irradiated Spisula sperm. The physical structure of the Spisula sperm must be such as to be incompatible with the Arbacia egg cortex.

It was noted, however, that Spisula spermatozoa irradiated to 63,000 r or more and kept for some 24 hours, even in the refrigerator, gave evidence of secretion of a mucilaginous substance which caused them to be clumped together into long strings (see Fig. 3). Such secretion and clumping did not inactivate the spermatozoa as they were seen still to be vibratile as if stuck by their tail tips.

Spisula eggs

A single specimen of this clam will produce literally millions of eggs (Allen, 1951) all of which are in the germinal vesicle stage and each of which measures about 50 microns in diameter (see Fig. 1). If the eggs are properly washed at least twice, they can be used for as long as 8–10 hours at laboratory temperatures or up to 24 hours if kept in the refrigerator at 10 degrees C. Even after 48 hours at refrigeration about 40% of these eggs can be fertilized and will develop into normal larvae (trochophores).

In general the eggs of Spisula are more radiosensitive than are the spermatozoa, when measured by the ability to cleave and to develop into trochophores. An exposure of the eggs to 6,300 r is roughly equivalent to 18,900 r to the spermatozoa. Since motility cannot be a criterion of activity, the only clue of the intact egg to damage is its failure to be fertilized or to develop normally.

One might expect that the egg, with its abundant cytoplasm, would show damage more readily than would the spermatozoon which is almost devoid of cytoplasm. However a few eggs exposed to 252,000 r did cleave following fertilization with normal spermatozoa but none developed into any semblance of a larval trochophore. There is evidence of membrane and cytoplasmic damage in these eggs because most of them became ruptured and developed vesicular protrusions following fertilization with normal sperm. (Compare Figs. 1 and 2.)

X-irradiated eggs showed cleavage delay when fertilized by normal spermatozoa but the delay was more linear than when x-irradiated sperm were used to fertilize normal eggs. Also, the eggs did not tolerate the magnitude of doses used for the

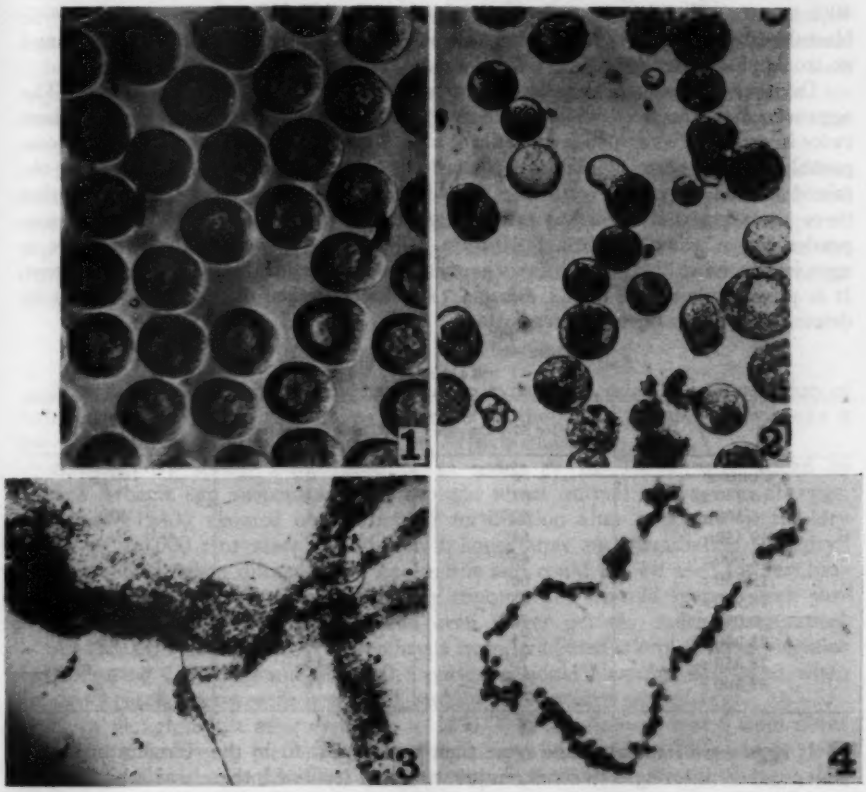

FIGURE 1. Normal eggs of Spisula, washed and ready for fertilization.

FIGURE 2. Spisula eggs exposed to 214,200 r x-rays and then fertilized by normal Spisula sperm, showing rupture and exovate masses. Some of the extruded masses and vesicles have rounded out to appear as miniature and deformed egg masses. Very few of these eggs ever cleaved, and none reached the trochophore stage.

FIGURE 3. Spisula sperm x-irradiated in cysteine which accentuated the secretion of a mucilaginous substance, causing the sperm to be aggregated into long strings.

FIGURE 4. Stringy aggregations of Spisula eggs x-irradiated in cysteine in sea water; reaction similar to that for spermatozoa (Fig. 3).

sperm. These data for the eggs include all eggs exposed but in the case of the spermatozoa it is impossible to determine what percentage of the invisible gametes are functional. At 3,150 r all eggs could be fertilized by normal sperm and would give rise to trochophores but at 31,500 r only some 70% of the eggs reached that

stage, even though there was 100% cleavage. The time of cleavage of 50% of x-irradiated eggs (fertilized by normal sperm) was as shown in Table III.

Spisula eggs exposed to higher levels of x-irradiation were able to cleave and develop. For instance, 90% of the eggs exposed to 94,500 r were fertilized and 40% became ciliated and motile trochophores. At 157,500 r there were also 90% blastulae and 10% trochophores and at 189,000 r there were very few cleavages and no trochophores developed.

Dilution of Spisula eggs had no appreciable effect on their radiosensitivity. The eggs were not irradiated while in the ovary because it was necessary to wash them twice in order to have them fertilizable at all. This meant that a concentration comparable to "dry sperm" (meaning gametes without any extra fluid) could not be obtained for the eggs. Nevertheless, eggs were concentrated after washing so that there was approximately a 1 : 1 ratio of eggs to sea water, and other eggs were suspended in sea water to give a 1 : 10 ratio. There was no appreciable difference in eggs irradiated in the two dilutions as measured by fertilizability and development. It is possible that even a 1 : 1 dilution (*i.e.*, the presence of any fluid) might be deleterious to eggs during x-irradiation.

TABLE III

	Time for 50% cleavage	Delay in minutes	Trochophores at 24 hours
Control	58″	0	100%
3,150 r	61″	3	
6,300 r	62″	4	60%
9,450 r	64″	6	
12,600 r	65″	7	15%
15,750 r	66″	8	
18,900 r	68″	10	5–10%
22,050 r	70″	12	
25,200 r	71″	13	10%
28,350 r	73″	15	
31,500 r	74″	16	5%

If eggs were irradiated and were then kept at 10° C. in the refrigerator for 24 hours and were brought to room temperature and fertilized, there was a higher percentage of cleavage and development to the trochophore stage than in eggs fertilized immediately after irradiation. It is not clear whether it was the delay or the refrigeration (or, possibly both factors) that seemed to be beneficial to the eggs. At 31,500 r some 40% ciliated trochophores appeared by 18 hours post-fertilization and at 63,000 r plus refrigeration some 50% trochophores developed. This may indicate some recovery on the part of the eggs under refrigeration, or it may indicate that toxic substances produced during irradiation in the medium in which the eggs were suspended had been dissipated.

There was some evidence of nuclear damage by exposure to 6,300 r since only about 60% of the eggs at this level reached the trochophore stage. The nuclear damage must have been most severe at 31,500 r (with 5% trochophore development) because as many as 40% trochophores again appeared following 94,500 r. Since further irradiation above 94,000 r did not improve trochophore production,

even though the change was still high, it may be presumed that cytoplasmic damage may have complicated the situation so that development could not proceed.

Spisula eggs exposed to various doses of x-irradiation were then submitted to normal spermatozoa of the echinoderm, Arbacia, pre-tested against eggs of Arbacia. At no level was a single irradiated Spisula egg fertilized by an Arbacia spermatozoon. At 264,600 r the Spisula eggs are ruptured by fertilization with normal Spisula sperm and develop ex-ovate masses, and disintegrate. If, however, such eggs are exposed to Arbacia sperm, these abnormal conditions do not occur, indicating that the Arabacia sperm are unable to penetrate the membrane and cortex even of these moribund eggs of Spisula.

In contrast with the irradiation of Spisula sperm, therefore, the egg is less tolerant and at the maximum exposure used (264,600 r) the egg membrane and cytoplasm were so damaged that fertilization was virtually impossible. The sperm, exposed to this dose, fertilized normal eggs to nearly 100%. With respect to the trochophores developing from irradiated sperm or irradiated eggs, there was no detectible difference.

Observations on Arbacia Gametes

Henshaw (1940) long since completed an exhaustive study of x-irradiation of Arbacia gametes. Nevertheless, as a parallel to the above Spisula experiments a few additional observations were made on Arbacia material.

Arbacia dry sperm x-irradiated to as much as 189,000 r were added to normal Arbacia egg suspensions and brought about normal cleavage in all eggs. Henshaw (1940) claimed obliteration of fertilization after exposure of the dry sperm to 300,000 r or more. However, 6 hours after such irradiation the "dry" Arbacia sperm, when diluted, were non-motile and could not be used for fertilization. This was not true for the similarly concentrated Spisula sperm which survived many hours post-irradiation, and even longer periods under refrigeration.

At 126,000 r about 50% of the embryos from irradiated sperm became blastulae and following 189,000 r and 252,000 r only occasional blastulae developed when the dry Arbacia sperm were irradiated, and used to fertilize normal eggs.

Even though motile after exposure to 189,000 r, the Arbacia sperm were never successful in fertilizing (activating) the normal eggs of Spisula. This interphyletic cross by irradiated sperm has not been attempted previously. It was thought that such sperm, with their genetic complement presumably damaged, might act as parthenogenetic agents and stimulate Spisula eggs at least to membrane elevation and cleavage. This, apparently, does not occur so that other incompatibilities must be present.

The cleavage delay following irradiation of Arbacia eggs (first reported by Henshaw, 1932 and Henshaw and Francis, 1933) was clearly substantiated. Further, Arbacia eggs exposed to x-rays at any level up to 126,000 r could not be fertilized by normal Spisula sperm. Again this suggests incompatibility apart from nuclear considerations since the irradiated nuclear material of the Arbacia egg was never reached by the normal Spisula sperm. Control situations, in which normal Spisula sperm were used with normal Arbacia eggs, never gave cleavage either. It was presumed that irradiated Arbacia eggs might have lost some of their resistance to foreign sperm. This was not borne out.

Arbacia plutei exposed to as little as 9,450 r very quickly aggregated into sticky clumps. This stickiness occurred soon after irradiation but did not last very long. It was probably due to a secretion from the irradiated plutei, for a similar stickiness has been reported for testicular chromosomes in other aquatic forms (Rugh, 1950). Plutei tolerated as much as 44,100 r with no more deleterious effects than a retardation in growth over a period of 7 days. The pluteus is therefore much less sensitive than is either gamete.

The original incentive for these studies was to determine whether chemical agents, previously demonstrated as having some protective value for mice against x-irradiation damage, might likewise have some protective effect on marine gametes and early embryos. The substances used were cysteine hydrochloride (Patt *et al.*, 1949, 1950) and another − SH compound known as B-mercaptoethylamine (Bacq *et al.*, 1951). Both substances have been reported to give protection to mice by injection before irradiation.

Cysteine

It was soon found that 0.5% cysteine was hypertonic and toxic to the eggs of Spisula; that a 0.1% solution allowed fertilization and cleavage but caused developmental abnormalities and that a 0.01% solution was toxic to developmental stages over extended periods but was not toxic for short periods to the gametes and early embryos of Arbacia. Fertilization could be accomplished to a normal degree in such a concentration of cysteine. Since exposure to cysteine was of rather short duration before, during and sometimes after irradiation, the concentration of 0.01% was used throughout the experiments.

Spisula eggs immersed in a 0.01% solution of cysteine in sea water (adjusted to pH 8.0) for as much as three hours before and during x-irradiation, had no effect whatever on fertilizability of the eggs, nor on the cleavage and early development. That is, both cleavage percentage and time were normal, similar to the controls which did not have the exposure to cysteine but did have the irradiation. Those eggs which were left in the solution in which they were irradiated, or were transferred to fresh cysteine, did not benefit by the presence of the cysteine with respect to developmental rate or degree when compared with the non-cysteine controls. In fact, if left more than a few hours no Spisula embryos reached the trochophore stage even though some of the non-irradiated controls did. Only when the cysteine concentration was reduced to 0.001% was there larval survival, but never to a degree of better than the non-cysteine controls. Exposure to cysteine before, during and after irradiation for a total of 1¼ hours did not affect later trochophore development. Eggs or embryos placed in previously irradiated cysteine were not thereby protected.

There was one bit of evidence of some cysteine effect. As stated above, when gametes or the larvae are x-irradiated to certain levels, they exude a sticky substance which causes them to aggregate (Figs. 3 and 4). This tendency is accentuated by the presence of cysteine. However, cysteine alone does not cause this and while x-irradiation does, the stickness is much more extensive when living material is irradiated in the cysteine medium. Further, a lower exposure (6,300 r) of the eggs or plutei to x-rays causes the stickiness in cysteine while it does not do

so without the cysteine. The aggregations under x-rays alone are in small clumps at first. They appear as long and heavy strings in x-ray plus cysteine conditions.

While cysteine does not have any effect on cleavage time or percentage, and it does not in any way seem to protect the gametes or early embryo against x-radiation, its presence does seem to cause a rounding out of the blastomeres of the two- and four-cell stages of both Spisula and Arbacia, stretching the intercellular connections so as to make them appear as though the blastomeres are almost unrelated. Many of the two-cell stages are almost separated. This is a situation one finds experimentally when such eggs are kept in a calcium-free or low-calcium medium.

B-Mercaptoethylamine (Bacq Solution)

This synthetic solution has a pH of 7.5 which is very close to that of normal sea water. It was adjusted to pH 8 before use. Bacq used 3 mg. per 30 gm. mouse, injected intraperitoneally, to give protection. This changed mortality of mice exposed to 700 r x-rays from 93% to 5%. When diluted to 0.1 mg. % in filtered sea water it was found to be lethal to Arbacia plutei; at 0.04 mg. % it was not toxic to the plutei but was toxic to the unfertilized eggs, causing a cortical wrinkling. The concentration which was not toxic, and which was finally used, was 0.01 mg. % at pH 8.0.

There was some slight statistical evidence that this solution allowed more eggs to develop and to develop further, following irradiation in the solution, than did the control situation of irradiation in sea water alone. There was also some slight evidence of acceleration of early cleavage. These were gross impressions which would have to be checked under highly controlled conditions of temperature and concentrations. Even then they would not be very significant, if confirmed, because the protection, if any, was so slight.

For instance, Arbacia eggs irradiated in the Bacq solutions did show a slightly higher percentage of development and the larvae developed farther than in the irradiated sea water controls. Also, Spisula eggs exposed to 18,900 r in the Bacq solutions survived to the trochophore stage better and in larger number than did their controls. But the degree of improvement in either case did not encourage an exhaustive statistical study since it was positive but not significant. One might say that if either solution showed any protection during irradiation it was the Bacq solution. In neither solution was development allowed to proceed normally very far.

Summary and Conclusions

1. The spermatozoa of the clam Spisula are more radioresistant than are its eggs.

2. The fertilizing power of Spisula sperm in the dry or concentrated state could not be affected by x-irradiation even to 264,000 r.

3. Increasing x-irradiation of Spisula spermatozoa caused increasing delay in cleavage time of normal eggs fertilized by such sperm, but the curve was not linear and did not exceed 15 minutes. This delay represents the time interval between the first and the second normal cleavages.

4. Trochophore production was at its lowest following 163,000 r x-irradiation of the spermatozoa, but with further x-irradiation trochophore production reached 95%. Such trochophores, while viable for a time, were not normal.

5. Dilution of Spisula spermatozoa increased their radiosensitivity as determined by the effect on subsequent embryonic development.

6. The demonstrated parthenogenetic stimulating ability of Spisula spermatozoa exposed to 189,000 r or more x-rays and used with Spisula eggs, could not be achieved when Spisula sperm were used with Arbacia eggs.

7. X-irradiated Spisula gametes exude a mucilaginous substance which causes them to aggregate (clump).

8. Very few Spisula eggs exposed to 189,000 r x-rays cleaved and none became trochophores. After 214,200 r there was evidence of cytoplasmic and membrane destruction.

9. Neither cysteine hydrochloride or B-mercaptoethylamine appeared to give any appreciable protection to Spisula gametes against x-irradiation as determined by the subsequent development of the embryo.

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PHYSIOLOGICAL ANALYSIS OF THE CORTICAL RESPONSE OF THE SEA URCHIN EGG TO STIMULATING REAGENTS. I. RESPONSE TO SODIUM CHOLEINATE AND WASP-VENOM

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The importance of the cortical response of sea urchin eggs in the process of fertilization or artificial activation has long been recognized and attention has been devoted to this problem. Loeb (1913) stated that the initiation of development in the sea urchin egg is due to a change in the surface of the egg. He maintained the theory, also, that cytolysis of the cortical layer generally results in membrane formation. According to Just (1919), some cortical change, beginning at the point of sperm-entry, sweeps over the egg before the actual elevation of the fertilization membrane. Gray (1922) postulated that the essential change of the egg surface is complete before any visible change is possible. Moser (1939) has found that after fertilization, the cortical granules embedded in the cortex of the Arbacia egg start to break down in a wave-like fashion and cause the formation of the fertilization membrane. He suggests that the breakdown of the cortical granules follows a preliminary release of calcium which may travel in a rapid wave-like fashion around the egg cortex. More recently, many interesting facts as to fertilization and activation are being found by the investigators of Runnström's institute.

However, despite great advances in our knowledge on this problem, it cannot yet be said that we have any sufficient understanding of the process of fertilization and activation. The present paper is the first of a series presenting the results of experiments which have been made in the hope of analyzing further the cortical response of the sea urchin egg.

Material and Methods

The sea urchin chosen for these experiments was *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus*, the spawning season of which extends over the whole winter. This species was used because the cortical granules of the egg are easily visible in the living state without centrifuging. With regard to the cortical granules of this species, Motomura (1941) studied them in detail, designating them "Janus green granules."

The eggs were secured in the usual manner. In all experiments only such egg material as showed good fertilizability in control experiments was used. For the first series of experiments a 1 per cent solution of sodium choleinate (Grübler) was made with sea water, sufficient amounts being added to sea water to make the desired solutions. The stock solution became ineffective in a few days but could be used for at least two days at room temperature (10–13° C.). The pH of the

sodium choleinate solution was 8.1–8.3. For the second series of experiments, wasp-venom was obtained from *Polistes fadwigae*. Cutting the abdomen of the wasp, the poison gland was taken out and dried in a desiccator. At the time of an experiment, it was placed in a few drops of sea water and venom was extracted. The concentration of wasp-venom was difficult to determine. All the experiments were made at room temperature.

Experiments with Sodium Choleinate

First, some typical experiments in which sodium choleinate was used to induce the formation of the fertilization membrane will be reported. Unfertilized eggs were put into solutions of sodium choleinate of various concentrations and their response was observed. It was found that sodium choleinate was effective in inducing the formation of the fertilization membrane, and the mode of response of the eggs was somewhat different from that obtained in experiments with butyric acid or urea solution. One of the results of the experiments is shown in Table I.

Table I

Formation of the fertilization membrane by means of sodium choleinate (temp. 11° C.)

Number of drops of Na-choleinate solution added to 1 cc. of sea water	Mode of membrane formation
20	The membrane is violently separated in 10 seconds (Fig. 1, a).
15	The membrane is violently separated in 40 seconds (Fig. 1, a).
10	"Normal" membrane formation took place in 50% of the eggs within 2 minutes, while some eggs formed only partially (Fig. 1, b, c). After 3 minutes, 92% of the eggs had completed membrane elevation.
5	No response was observed in most of the eggs after 5 minutes. Partial membrane formation occurred in a few eggs (Fig. 1, c).
4	No response was observed in any of the eggs after 5 minutes.

At the optimum concentration of sodium choleinate, formation of a normal-appearing fertilization membrane occurred within a few minutes. However, the mode of membrane elevation was rather different from that which takes place when the eggs are treated with butyric acid or urea solution. When such eggs are returned to sea water, roughening of the whole surface takes place within several seconds. But in the experiments with sodium choleinate, roughening of the whole surface of the eggs did not occur before membrane elevation. The membrane began to rise in one or several places, and a few minutes were required to complete its full elevation.

Too high concentration of sodium choleinate resulted in violent separation of the membrane and subsequent cytolysis. When a solution of sodium choleinate of too low concentration was used, no response was observed in most of the eggs, but partial separation of the membrane sometimes took place in a few eggs. Such par-

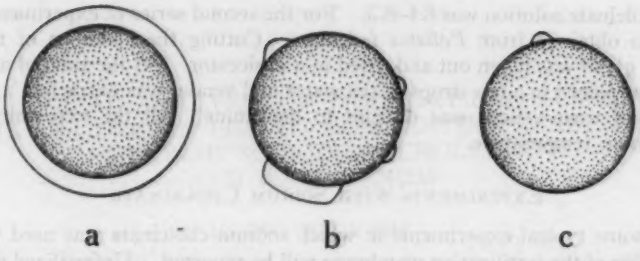

FIGURE 1. Eggs treated with sodium choleinate. a, an egg with completely elevated membrane; b and c, eggs with partially elevated membranes.

tially separated membranes remained as such even after twenty minutes' immersion in the solution.

In the next experiments, eggs treated with sodium choleinate solution of the optimum concentration (1 cc. of sea water plus 10 drops of 1% sodium choleinate solution) were washed with normal sea water when a partial separation of the membrane had just taken place. It was found that in such eggs membrane separation stopped and no further progress of the cortical change was observed. Upon examining the eggs with the oil immersion objective, it became obvious that the breakdown of the cortical granules occurred only in the part where the membrane had separated and the granules were intact elsewhere (Fig. 2). It was also found that when such eggs were inseminated after several washings with sea water, fertilization took place and the membrane separated all over the egg surface within a few minutes. The cortical granules of these eggs were thoroughly broken down. Most of these eggs, however, showed polyspermic development.

When unfertilized eggs are insufficiently treated with butyric acid, the fertilization membrane is sometimes elevated eccentrically or very slightly, but such a

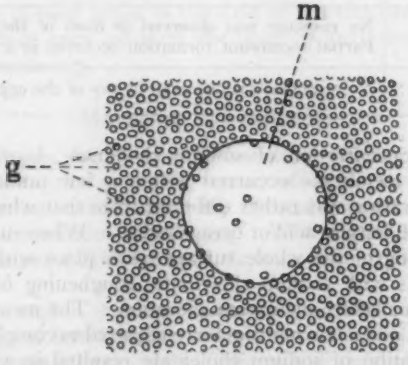

FIGURE 2. The cortex of the egg with partially elevated membrane. The cortical granules are broken down only in the area where the membrane is elevated. g, cortical granules; m, partially elevated membrane.

strictly partial separation of the membrane as in the case of sodium choleinate seldom occurs. It is interesting that the cortical response to an insufficient treatment with sodium choleinate is somewhat different from that to an insufficient treatment with butyric acid.

Experiments with Wasp-venom

Öhman (1944) reported that a membrane similar to a normal fertilization membrane was formed under the action of bee-venom. The experiments with bee-venom have thereafter been repeated and extended by Runnström (1947), and interesting results, especially concerning the behavior of the cortical material, have been obtained.

The present author has found that there is something in common between the actions of wasp-venom and sodium choleinate. If the unfertilized eggs of *Hemicentrotus pulcherrimus* are exposed to the action of appropriate concentrations of wasp-venom in sea water, small blisters appear at several places on the egg surface and soon an elevation of the membrane begins. If the action of the wasp-venom is continued longer, a complete membrane is formed in a few minutes. However, if the eggs are washed with sea water when an elevation of the membrane has occurred only locally and not over the whole egg surface, the membrane formation is interrupted as in the experiments with sodium choleinate. If these are observed with the oil immersion objective, it is found that the cortical granules have been broken down in the part of the egg cortex where the membrane is separated, while they are intact in the remaining areas.

Runnström has reported that when the treatment with bee-venom was interrupted by washing with sea water the membrane formation in Psammechinus eggs took place almost to the same extent as under continuous action of the bee-venom. The same results were obtained in Hemicentrotus eggs also, when a high concentration of wasp-venom was used. But a partial elevation of the membrane took place when an appropriate concentration of wasp-venom was used.

Discussion

It is well known that the first remarkable change of the sea urchin egg at the time of fertilization is, in general, a roughening process at the surface. Moser (1939) found in Arbacia eggs that this phenomenon is a consequence of the breakdown of the cortical granules. He found also that in from 10 to 20 seconds after insemination the cortical granules start to break down in a wave-like fashion, beginning at the site of sperm-entry and ending at the opposite pole of the egg. A similar behavior of the cortical granules is also observed in Hemicentrotus eggs (Motomura, 1941).

It has been suggested by several workers that some sort of invisible change is complete before any visible change occurs at the time of fertilization. This suggestion has generally been made on the basis of the fact that the block to polyspermy is rapidly established with the attachment or entry of one spermatozoon. According to Just, for instance, the "wave of negativity" progressively sweeps over the egg from the point of sperm-entry, preceding the actual beginning of membrane lifting, so that before the membrane begins to lift sperm can no longer enter at any point of the egg surface. It might be postulated that the breakdown of the cortical

granules follows the so-called wave of negativity. But there has been no evidence to prove causality between them. There may be some possibility that the wave of negativity is not connected with the breakdown of the cortical granules.

If we assume that the breakdown of one cortical granule does not automatically induce the breakdown of its neighbours, then the granule breakdown must be the consequence of a more profound cortical change of a self-propagating nature. The results presented in this paper make it possible to discuss some problems on the process of the breakdown of the cortical granules. If eggs treated with sodium choleinate or wasp-venom are washed with sea water when elevation of the membrane has occurred only at several places on the egg surface, the membrane formation is interrupted. It has been noted that the cortical granules are broken down in the parts of the egg cortex from which the membrane is separated, while they are intact in the remaining areas. It is evident that the failure of the granular breakdown is not due to some impairing effect of the reagents, because, as stated above, the remaining granules are broken down if insemination is carried out afterwards. These facts constitute the evidence for concluding that the breakdown of the cortical granules itself at any point does not become the cause of the breakdown of the neighbouring granules. It seems quite possible, therefore, that some invisible cortical change of a propagating nature must occur in order that the cortical granules are broken down at the time of fertilization. Whether such an invisible cortical change is identical with Just's "wave of negativity" or not, is still obscure, because there has been no evidence to indicate that the mechanism to exclude supernumerary spermatozoa is the cause of breakdown of the cortical granules.

The work of Yamamoto (1944a, 1944b) on the eggs of the fish, *Oryzias latipes*, has demonstrated that the first visible change at fertilization is the wave-like breakdown of alveoli which are embedded in the cortical protoplasm of ripe unfertilized eggs. He postulated that a reaction of some kind, or an impulse, may be provoked by the sperm and conducted in a wave-like fashion, causing such alveolar breakdown. He termed this invisible wave the "fertilization-wave." The Oryzias egg is much larger than the sea urchin egg and its structure and nature differ from those of the sea urchin egg to a considerable degree. But the processes of the fertilization reaction in the two animals seem to have something in common with each other. The invisible cortical change in the sea urchin egg resembles the "fertilization-wave" in Oryzias eggs in its self-propagating nature, in its occurrence prior to the breakdown of the cortical structure and in its function of causing the breakdown of the cortical structure. Therefore, the author wishes to call the invisible cortical change in sea urchin eggs, also, the "wave of fertilization" or the "fertilization-wave."

As suggested elsewhere in this article, the cortical response to an insufficient treatment with sodium choleinate is somewhat different from that to an insufficient treatment with butyric acid. By means of sodium choleinate a partial separation of the membrane can be induced. This suggests that sodium choleinate causes the breakdown of the cortical granules without intervention of the fertilization-wave. This suggestion is also applicable to wasp-venom. On the other hand, such a strictly partial separation of the membrane as in the case of sodium choleinate seldom occurs as the result of an insufficient treatment with butyric acid. This probably indicates that butyric acid gives rise to the fertilization-wave, indirectly inducing the breakdown of the cortical granules. The data seem insufficient to war-

rant a more extended analysis, but indicate the desirability of a more refined approach to the problem of the response of the eggs to parthenogenetic reagents.

The author wishes to express his gratitude to Prof. T. Yamamoto, Dr. Jean C. Dan and Mr. E. Nakano for their valuable suggestions and advice concerning the experiments and the manuscript.

Summary

1. The formation of the fertilization membrane in Hemicentrotus eggs can be induced by an appropriate treatment with sodium choleinate or wasp-venom. When the concentration is optimum, the membrane begins to rise in one or several regions of the egg surface and several minutes are required to complete its full elevation.

2. If the eggs are washed with sea water when elevation of the membrane has occurred only locally and not over the whole egg surface, the membrane formation is interrupted. The cortical granules have been broken down only in the part of the egg cortex where the membrane is separated while they are intact in the remaining areas.

3. These results constitute the evidence for the conclusion that the breakdown of the cortical granules itself at any point does not become the cause of the breakdown of the neighbouring granules. It seems to follow, therefore, that some invisible cortical change of a propagating nature must occur in order that the cortical granules be broken down at the time of fertilization. This invisible cortical change may be called the "wave of fertilization" or the "fertilization-wave," since it resembles Yamamoto's "fertilization-wave" in Oryzias eggs.

4. It is suggested that sodium choleinate and wasp-venom cause the breakdown of the cortical granules without intervention of the fertilization-wave.

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PHYSIOLOGICAL ANALYSIS OF THE CORTICAL RESPONSE OF THE SEA URCHIN EGG TO STIMULATING REAGENTS. II. THE PROPAGATING OR NON-PROPAGATING NATURE OF THE CORTICAL CHANGES INDUCED BY VARIOUS REAGENTS

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In the previous paper of this series, it was concluded that an invisible change of a propagating nature must occur at the time of fertilization in sea urchin eggs in order that the cortical granules be broken down. This change was called the "fertilization-wave," as in the case of Oryzias eggs (Yamamoto, 1944). It was suggested that sodium choleinate and wasp-venom do not initiate the fertilization-wave, although they exert their influence upon the cortical granules to induce their breakdown. This means that the artificially induced breakdown of the granules does not always reveal itself as a consequence of the fertilization-wave. The question then arises as to the presence or absence of the fertilization-wave in the cortical changes induced by various other artificial stimuli. The possibility must be considered that some stimulating reagents provoke the fertilization-wave, which leads to the breakdown of the cortical granules, while others act directly to cause the breakdown of the cortical granules, without inducing a fertilization-wave.

An investigation has been made to determine whether the effects of various stimulating reagents are of a propagating nature. If they are of a propagating nature, it may very probably indicate that the fertilization-wave is provoked. On the contrary, if they are of a non-propagating nature, it may be clear that the fertilization-wave does not occur. It is the purpose of the present paper to report the results of these experiments.[1]

Material and Methods

The sea urchin, *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus* was used throughout. The eggs to be used for experiments were carefully collected in the usual manner and only such eggs as showed good fertilizability in control experiments were used. Wasp-venom, sodium choleinate, urea, glycerine, sucrose, distilled water, detergents and fatty acids were employed as stimulating reagents.

In order to investigate whether the effect of a stimulating reagent is of a propagating nature, the following method was adopted. The egg surface was partially exposed, as described below, to the stimulating reagent for an appropriate time and then washed with sea water. The unexposed surface was examined with the

[1] It may be added that elaborate works on conduction of the block to polyspermy have been done by Rothschild and Swann (*Exp. Cell Research*, **2**: 137, 1951; *J. Exp. Biol.*, **28**: 403–416, 1951; *J. Exp. Biol.*, **29**: 469–483, 1952).

oil-immersion objective. When the cortical granules in the exposed part were broken down and those in the unexposed part were intact, it was considered that the effect of the reagent was not of a propagating nature. But when the cortical granules in the whole surface, including the unexposed part, were broken down, the effect of the reagent was conceived to be of a propagating nature.

Partial exposure of the egg surface to the reagent was achieved by means of the technique diagrammed in Figure 1a. The jelly-coats of the eggs were first removed by treating the eggs with a weak solution of HCl in sea water. It was

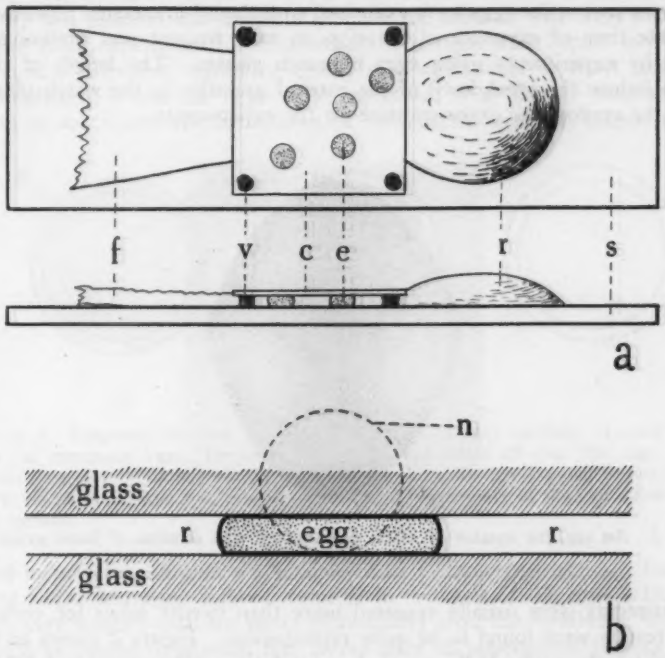

FIGURE 1. a, Diagrammatic view of the arrangement used for the experiments. b, Diagrammatic view of the compressed egg. c, cover-glass; e, egg; f, filter-paper; n, outline of a normal egg; r, reagent to stimulate eggs; s, slide-glass; v, vaseline.

ascertained that the eggs thus deprived of their jelly-coats were fertilizable. A small drop of sea water containing the jelly-less eggs was placed on a slide. A very small amount of vaseline was put on the four corners of a cover-glass and this was placed over the egg-sea water drop. Pushing the cover-glass with a fine needle, the eggs were pressed down and flattened until their diameter reached 160 μ. Since the normal spherical egg has a diameter of about 98 μ, the flattened eggs were considered to take the form schematized in Figure 1b. The upper and lower parts of the eggs adhered closely to the glass-surface, while the equatorial region, so to speak, was in contact with the sea water. It was found possible to control

the amount of vaseline so that there was no movement of the eggs during the subsequent procedure.

Several drops of the reagent to be tested were placed on the slide at one side of the cover-glass (Fig. 1a). A small piece of filter paper was applied to the other side of the cover-glass so that the reagent was drawn into the space beneath the cover-glass. After an appropriate time, the reagent was replaced by normal sea water in a similar way. Thus the equatorial region of the eggs was exposed to the reagent, while the upper and lower regions were not influenced directly by the reagent, since they were closely applied to the glass-surfaces. The cortical granules in the upper part of the eggs were examined with the oil-immersion objective. The appropriate time of exposure of the eggs to each reagent was preliminarily determined by experiments using eggs in watch glasses. The length of time sufficient to induce the breakdown of the cortical granules in the watch glasses was taken as the appropriate exposure time for the experiments.

FIGURE 2. An egg, the equator of which was exposed to a solution of Janus green by the method shown in Figure 1.

Experiments were usually repeated more than twenty times for each reagent and the results were found to be quite reproducible. Figure 2 shows an egg, the surface of which was partially exposed to a solution of Janus green by the above-described method. The equatorial region is deeply stained while the other areas remain unstained, indicating that the dye did not soak into these regions. The fact that, as will be shown later, even surface-active substances such as detergents do not exert their influence on the upper part of the eggs indicates that this technique is suitable for the present purpose.

RESULTS

Experiments with wasp-venom, sodium choleinate and detergents

Wasp-venom was obtained from *Polistes fadwigae*. The poison gland was placed in a few drops of sea water and the venom was extracted. Employing the above-described technique, the equatorial regions of the eggs were exposed to the

solution of wasp-venom in sea water. With an appropriate concentration of wasp-venom, the cortical granules in the equatorial region soon began to break down. After they had completely broken down, the upper part of the eggs was examined. It was found that the cortical granules in that part were quite intact and no sign of the influence of the wasp-venom was observed (Fig. 3b). If the solution was replaced by normal sea water after the membrane was elevated on the equatorial region and before cytolysis took place, no cortical change was observed in the upper part of the eggs even after 10 minutes.

Similar experiments were performed using sodium choleinate. To 1 cc. of sea water were added 10 drops of a 1 per cent solution of sodium choleinate. The result with this solution was found to be quite the same as that with wasp-venom.

In the next experiments, the effects of Monogen and Lipon were studied. Monogen is a detergent, consisting chiefly of a mixture of myristyl sulphate and lauryl sulphate. When the eggs were immersed in a 0.5 per cent solution of Monogen in sea water, the breakdown of the cortical granules began in a short

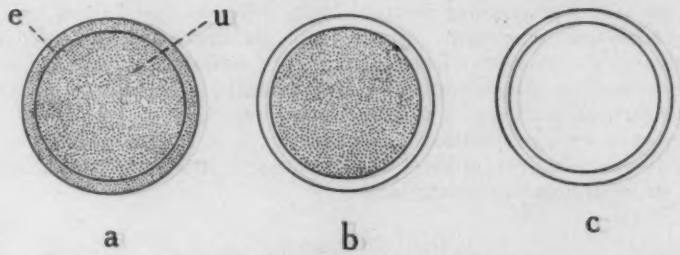

FIGURE 3. Diagrams to show the cortical response of eggs partially exposed to various reagents. a, unexposed egg. The cortical granules are intact all over the egg. b, an egg exposed to wasp-venom, sodium choleinate or Monogen. c, an egg exposed to fatty acid, non-electrolyte or distilled water. e, equatorial region; u, upper part of the egg adhering closely to glass surface.

time and membrane elevation was completed in 2 minutes at 18° C. Too long an exposure to the Monogen solution resulted in cytolysis of the eggs. However, if the eggs were washed with sea water immediately after the membrane was formed, they could be caused to develop into larvae after an appropriate treatment with a hypertonic solution. Thus, Monogen has proved to be an excellent parthenogenetic agent. Lipon, another detergent, consisting chiefly of alkyl sulfonate, has a similar effect. It was found that the result of partial exposure of the egg surface to Monogen or Lipon was almost the same as those with wasp-venom and sodium choleinate.

These results suggest that the cortical change caused by treatment with wasp-venom, sodium choleinate, Monogen and Lipon is not of a propagating nature.

Experiments with fatty acids, non-electrolytes and distilled water

Monobasic fatty acids are known to be very effective in inducing membrane formation in sea urchin eggs. Experiments were performed employing the technique

described above, in order to determine whether the effect of fatty acids is of a propagating nature. The egg surface was partially exposed to butyric acid sea water (47 cc. of sea water plus 3 cc. of N/10 butyric acid) for 30 seconds at 18° C., and then the solution was replaced by normal sea water. After about 20 seconds the cortical granules in the equatorial region of the egg began to break down and a membrane started to separate. The granular breakdown then rapidly proceeded in the upper unexposed part of the egg and within 5 to 9 seconds their breakdown was finished all over the egg surface (Fig. 3c). Acetic and propionic acids also were found to have the same effect. It is apparent that these results are entirely different from those with wasp-venom, sodium choleinate and detergents. It is concluded that the effect of fatty acids is of a propagating nature.

It has been known since the publication of Motomura's work (1934) that urea solution is an excellent activating agent. Moser (1940) has observed that Arbacia eggs treated with molar concentrations of non-electrolyte solutions exhibit the same kind of visible cortical response as that obtained with sperm cells and other stimulating agents. Therefore, it is of interest to determine whether the effect of non-electrolytes is of a propagating nature. Molar solutions (pH 7.0) of urea, sucrose and glycerine were employed. Among these, the urea solution proved the most suitable, since almost 100% of the eggs could be activated. The experimental results were the same as those obtained with fatty acids, indicating that the effect of a non-electrolyte solution is of a propagating nature.

Another series of experiments was undertaken to study the effect of distilled water. It was found that distilled water also was a strong stimulating agent, having the same effect as that of fatty acids.

Discussion

Up to the present, numerous reagents have been found to be effective for inducing the elevation of the fertilization membrane in sea urchin eggs. The range of such effective reagents is exceedingly wide, but in every case the first visible cortical response, regardless of the nature of the reagent, is the breakdown of the cortical granules (Moser, 1939). The elevation of the membrane follows this initial visible cortical response. On the basis of these facts it has been believed by some workers that stimulating reagents other than sperm cells effect essentially the same type of cortical response as that obtained upon insemination. However, the results presented in this paper make it possible to classify the stimulating reagents into two groups according to the nature of their effects. This further means that their effects are not always the same so far as the invisible cortical response is concerned. The first group includes reagents such as butyric acid, distilled water and isotonic solutions of non-electrolytes. The cortical change provoked by these reagents is of a propagating nature. To the second group belong reagents such as wasp-venom, sodium choleinate, Monogen and Lipon. The response to their effects is of a non-propagating nature.

It should be noted here that the propagating nature of the change induced by reagents which belong to the first group is capable of proof only when the egg surface is partially exposed to the reagents. In usual experiments the eggs in any solution are completely exposed to it, so that the propagating nature of the response is not detectable. The question arises, therefore, as to the occurrence of the propa-

gation of the response in such cases. Yamamoto (1944) has shown in Oryzias that there is a gradient of irritability in the cortex of the unfertilized egg. According to him, the irritability is highest at the animal pole, medium at the equator and lowest at the vegetal pole. If it be assumed that there is a gradient of irritability in the cortex of the sea urchin egg also, and in addition that the response occurs first in the most irritable part of the cortex when the egg is immersed in a solution of a stimulating reagent, it would seem probable that the response might travel in a rapid wave-like fashion around the egg cortex before the direct response to the reagent occurred in the other part. At present, however, no evidence has been furnished as to such a gradient of irritability in the sea urchin egg. Therefore, there remains another possibility, that the cortical response in all parts of the egg surface takes place at the same time, being directly provoked by the chemical stimulus. However, regardless of this, the response induced by a reagent of the first group should be considered to be essentially different from that induced by reagents of the second group, since the former was able to propagate over a part of the egg surface not exposed to the reagent, while the latter was not.

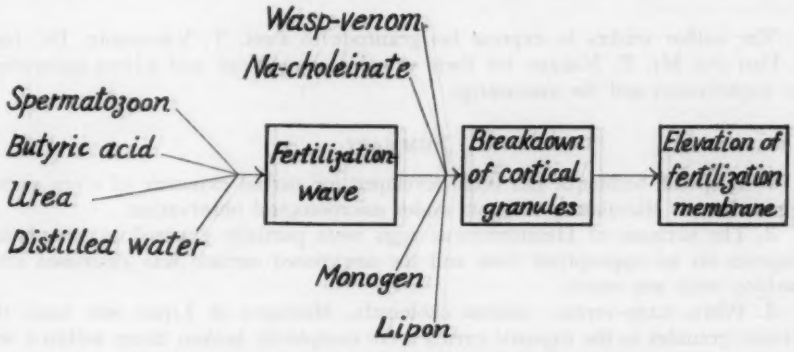

FIGURE 4. Diagram showing the process of the cortical change.

In the first paper of this series it was shown that the breakdown of one cortical granule does not automatically induce the breakdown of its neighbours. The author is therefore convinced that there must be an invisible wave-like change underlying the breakdown of the cortical granules whenever the cortical change is of a propagating nature. According to this concept, reagents such as urea or butyric acid stimulate the eggs to initiate an invisible wave-like change, which is followed by the breakdown of the cortical granules.

In the previous paper it was also postulated that some invisible cortical change of a propagating nature must occur at the time of fertilization. This invisible cortical change was called the "fertilization-wave." The nature of the cortical change provoked by the reagents of the first group discussed in this paper suggests that this change is essentially the same as that resulting from the entrance of spermatozoa. If this suggestion be true, then it may be said that the reagents of the first group induce the fertilization-wave itself, while the reagents of the second group in-

duce the same changes as the fertilization-wave, without the intervention of the fertilization-wave. This conclusion is essentially in agreement with the view considered on the effect of wasp-venom and sodium choleinate in the previous paper.

These concepts give us the scheme diagrammed in Figure 4. It shows that the spermatozoon and the reagents of the first group give rise to the fertilization-wave and this causes the breakdown of the cortical granules. The reagents of the second group cause the breakdown of the cortical granules without the intervention of the fertilization-wave. The granular breakdown, then, is followed by the elevation of the fertilization membrane, regardless of the nature of the activating reagent.

In a recent paper, Runnström and Kriszat (1952) reported that in Psammechinus eggs the propagation of the impulse caused by the attachment of the spermatozoon is inhibited by the attachment of the egg surface to glass. The data on Hemicentrotus eggs do not agree with these findings. Either there is a great difference in these two sea urchins with respect to the nature of the cortex, or the dissimilarities must be accounted for by differences in the conditions under which the observations were made.

The author wishes to express his gratitude to Prof. T. Yamamoto, Dr. Jean C. Dan and Mr. E. Nakano for their valuable suggestions and advice concerning the experiments and the manuscript.

Summary

1. A special technique has been developed for partial exposure of a sea urchin egg surface to stimulating reagents under microscopical observation.

2. The surfaces of Hemicentrotus eggs were partially exposed to stimulating reagents for an appropriate time and the unexposed surface was examined after washing with sea water.

3. When wasp-venom, sodium choleinate, Monogen or Lipon was used, the cortical granules in the exposed cortex were completely broken down within a few minutes, while those in the unexposed cortex remained quite intact, showing no sign of the influence of the reagent. The effect of these reagents is believed to be of a non-propagating nature.

4. When butyric acid, acetic acid, propionic acid, distilled water or an isotonic solution of a non-electrolyte was used, the granular breakdown proceeded rapidly in the unexposed part of the cortex immediately after granule breakdown in the exposed part. Therefore, it is concluded that these reagents induce a cortical change of a propagating nature.

5. It is suggested that the nature of the cortical change provoked by reagents of the latter group is essentially the same as that which follows the entrance of spermatozoa.

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PROLONGATION OF LIFE-SPAN OF SEA URCHIN SPERMATOZOA, AND IMPROVEMENT OF THE FERTILIZATION-REACTION, BY TREATMENT OF SPERMATOZOA AND EGGS WITH METAL-CHELATING AGENTS (AMINO ACIDS, VERSENE, DEDTC, OXINE, CUPRON)

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The addition of an amino acid to the sea water in which spermatozoa of sea urchins and other marine invertebrates are diluted has been found (Tyler, 1950; Tyler and Atkinson, 1950) to extend very considerably their functional life-span. By means of these agents the duration of fertilizable life and motility may be extended more than 50-fold. Treatment of the spermatozoa also has an effect on the nature of the fertilization reaction. Thus, eggs of Lytechinus often exhibit incomplete membrane elevation when fertilized by spermatozoa that have been diluted in sea water. In general this effect depends upon the extent of dilution and the age of the sperm suspensions. Treatment of the spermatozoa with an amino acid corrects this effect where it occurs with freshly diluted sperm and, parallel to the extension of fertilizable life and motility, extends the period during which a good fertilization-reaction is elicited. Runnström *et al.* (1946; *cf.*, Runnström, 1948; Wicklund and Gustafson, 1949) have reported that treatment of so-called underripe eggs of sea urchins with glycine and other amino acids and fertilization in these solutions improves their fertilizability and gives good membrane elevation. Tyler and Atkinson (1950) have also obtained this effect but note that pre-treatment of the eggs and inseminating in sea water gave much less improvement in fertilization and membrane elevation than obtained by treatment of the sperm.

In starfish another effect of amino acid treatment of the sperm was discovered by Metz and Donovan (1950). This consists in enabling the sperm to be agglutinated by the egg water of this species whereas ordinarily the agglutination reaction fails.

The extension of life-span of the sperm is accomplished with no apparent utilization of the amino acid. Thus determinations of glycine showed no significant loss during prolonged incubation with sperm, nor was there any significant production of ammonia (Tyler and Atkinson, 1950). Similar tests with carboxyl C^{14}-labelled glycine showed a negligible amount of decarboxylation (Tyler and Rothschild, 1951). The metabolism of the spermatozoa is, however, affected by the presence of the amino acid (Tyler and Rothschild, 1951). When sea urchin spermatozoa are diluted in ordinary sea water there is an initial great increase in rate of respiration followed by a decrease to barely measurable values accompanying loss of fertilizing

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capacity and motility. In the presence of amino acid the initial burst of oxygen uptake is suppressed, proportionately to the concentration of the amino acid. The rate then decreases to a relatively low but quite appreciable value where it remains rather constant during the extended life-span of the sperm. The total oxygen uptake of the treated sperm during its extended life span considerably exceeds that of the controls. From this, together with the evidence of non-utilization of the added amino acid it is clear that death of the sperm in ordinary sea water is not due to exhaustion of oxidizable substrate. The addition of amino acid evidently enables the spermatozoa to utilize more fully their endogenous substrate. Another interesting effect of the amino acid is to enable the spermatozoa to remain motile and retain fertilizing capacity under anaerobic conditions, whereas in ordinary sea water they die promptly in absence of oxygen.

In view of the above-mentioned evidence it was suggested (Tyler and Rothschild, 1951) that the amino acids act by virtue of their ability (*cf.*, Greenberg, 1951; Martell and Calvin, 1952) to bind certain heavy metals ordinarily present in sea water. This hypothesis is supported by the results of tests with other kinds of metal-chelating agents and with artificial sea water reported in the present paper.

Material and Methods

The animals used in these experiments were: the sea urchins *Arbacia punctulata, Strongylocentrotus purpuratus* and *Lytechinus pictus;* the sand dollar *Echinarachnius parma* and the polychaet *Chaetopterus pergamentaceus.* The eggs and sperm were obtained from the sea urchins and the sand dollars by the KCl-injection method (Tyler, 1949). From Chaetopterus they were obtained by excision of parapodia containing the ripe gonads.

The sperm concentrations are expressed as a percentage (1% and 0.1% in most of the experiments) of undiluted semen, the suspensions being prepared by direct dilution of the "dry" semen in the test solutions or immediately after a preliminary 5-fold dilution in sea water. "Dry" semen of the sea urchins *S. purpuratus* and *L. pictus* contains approximately 2×10^{10} spermatozoa per ml. The suspensions were kept at room temperature (19° to 22° C.) in Pyrex, 25 or 50 ml., stoppered Erlenmeyer flasks on a slow rocker (10 c.p.m. over an angle of 60°) or, in a few experiments, unshaken in a shallow (1 mm.) layer.

Fertilizing capacity was determined from the results of inseminating freshly collected eggs (ca. 500) in 4 ml. of sea water with various amounts (usually forming a 2-fold dilution series) of the sperm suspensions. The senescing experimental and control sperm suspensions are thus compared with each other, as well as with the freshly diluted semen, on the basis of the amount required to give the same percentage of fertilization, where this percentage is less than 100 and greater than 0. From this the relative duration of fertilizable life is obtained. Since fertilizing capacity tapers off gradually as the sperm suspensions approach the end of their life-span but shows a rather rapid decline around the 50 per cent value it was considered best to compare the suspensions on the basis of their half-life-spans. These are the figures given in Tables I and II. It should be noted, however, that on other bases of comparison such as "total" life-span or 10% life-span the results are qualitatively the same and the relative values do not differ very greatly.

For the motility determinations the intervals at which readings were taken

were determined on the basis of preliminary tests so that the usually sharp drop-off could be reasonably adequately covered. Motility was scored as 0, 1, 5, 25, 50, 75, 90 and 100 per cent. Again the duration of motility is expressed (Tables I and II) as a half-life. "Total" life-spans generally range some 50 to 100 per cent greater.

Sea water solutions of the various test substances were prepared from stock solutions made up with glass double-distilled water and adjusted to the pH of the sea water. Osmotic pressures were adjusted so that the most concentrated solutions employed did not deviate by more than 5 per cent from isotonicity with sea water. The sea water in most of the experiments was collected outside the laboratory at incoming tide and was filtered through washed "sharkskin" paper. It should be noted that it is important to check the pH of each solution made up by dilution of a stock solution in sea water. Thus, for example, when a stock solution of ethylenediaminetetraacetic acid at pH 8.2 is diluted with sea water to make a 10^{-3} molar solution there is a drop in pH to about 6.2. This is due to the displacement of protons from the weakly acidic ammonium groups of this compound as a result of chelation with some of the cations present in the sea water (*cf.*, Martell and Calvin, 1952).

Experiments

Life-span of spermatozoa in solutions of amino acids and peptides. In Table I the results of experiments with ten different amino acids and two peptides are summarized. All of these proved to be effective in extending both the fertilizable life and duration of motility of the spermatozoa. For glycine the optimum concentrations range around 0.05 and 0.1 molar. For the others the data are insufficient to specify optimum concentrations. The cysteine concentrations refer only to the initial solutions since this substance is rapidly oxidized to cystine upon preparation of the suspensions. The indications are that this may be effective in lower concentration than is glycine and the same may be true for glutathione.

The degree of extension of life-span obtained with these agents is dependent on the density of the sperm suspension. As is well known (*cf.*, Gray, 1928a, 1928b, 1931; Rothschild, 1948, 1951) the life-span of spermatozoa decreases with increasing dilution of the suspension. Comparison of the values of half-life-span in sea water (column headed 0) for the different semen concentrations, in experiments employing the same species, illustrates this dilution effect. In the amino acid solutions the "absolute" extension of life-span, in hours, is generally greater for the denser than the more dilute suspensions. The relative increase in life-span is, however, greater the more dilute the sperm suspension. Thus, in the experiments with Lytechinus listed in Table I the average half-life-spans for the 5, 1, 0.5 and 0.1 per cent sperm suspensions in 0.05 *M* glycine are, respectively, 7 to 12, 18 to 46, 50 to 80 and 100 times those for the sea water controls.

The present experiments also show a correlation between fertilizing capacity and motility. While it is well known (see Tyler, 1948; Rothschild, 1951 for references) that motile spermatozoa can be rendered non-fertilizing by various means, it is general experience that non-motile spermatozoa cannot effect fertilization. The present results indicate that loss of fertilizing capacity roughly parallels loss of motility.

Life-span of spermatozoa in solutions of other metal-chelating agents. In this category the effect of the following substances was investigated: ethylenediamine-

TABLE I

Action of amino acids on life-span of spermatozoa at 19°–22° C.

Substance	Species	Semen conc.	No. of experiments	Half-motile life* and half-fertilizable life† in: (concentration in sea water)						
				0	0.25 *M*	0.1 *M*	0.05 *M*	0.025 *M*	0.01 *M*	0.001 *M*
				hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
Glycine	*S. purp.*	1%	1*	2	48	53	27	7	5	1
		1%	1†	1	20	40	40	15	10	1
		0.1%	1*	0.5	26	31	17	2.5	0.5	1
		0.1%	1†	0.5	10	25	20	5	0.5	0.5
	L. pict.	5%	3*	1.5			18			
		5%	2†	2			15			
		1%	3*	1			18		1	1
		1%	1†	0.5			23			
		0.5%	8*	0.2			16			
		0.5%	3†	0.2			10			
		0.1%	2*	0.02	1.3	3	2		0.05	0.05
	A. punct.	1%	1*†	6–5			20			
		0.5%	1*†	2–1			49+			
		0.1%	1*†	0.5			4+			
		0.05%	2*†	<0.5			2.5+			
	E. parma	0.25%	1*†	1			8.5+			
	Ch. perg.	0.1%	1*†	0.3			4.5+			
Alanine	*L. pict.*	0.1%	1*†	0.02		1+				
	A. punct.	0.1%	1*†	1		24+				
Valine	*L. pict.*	0.1%	1*†	0.02		1+			0.02	
Leucine	*L. pict.*	0.1%	1*†	0.02		1+			0.02	
Lysine	*L. pict.*	0.1%	1*†	0.02		1+			0.02	
Glutamic	*L. pict.*	0.5%	1*	0.1			20+			
	A. punct.	0.1%	1*†	5			40+			
Histidine	*A. punct.*	0.5%	1*†	4			10+			
	A. punct.	0.1%	1*†	1.5			10+			
	A. punct.	0.01%	1*†	0.02			10+			
Phenylalanine	*A. punct.*	0.02%	1*†	0.02			3.5+			
	A. punct.	0.005%	1*†	0.02			3.5+			
Tryptophane	*A. punct.*	0.02%	1*†	0.02			3.5+			
	A. punct.	0.005%	1*†	0.02			3.5+			
Cysteine	*A. punct.*	0.05%	1*†	2			1		10+	10+
	A. punct.	0.01%	1*†	0.5			1		2	4
	A. punct.	0.002%	1*†	<0.02			0.5		2	2
Glycylglycine	*A. punct.*	0.5%	1*†	6			48+			
Glutathione	*L. pict.*	1%	1*	<0.4		3	2	2	1.4	0.5
	L. pict.	0.1%	1*	<0.3		1.8	2.5	1	1	0.3

tetraacetic acid (known commercially as Versene [2]), diethyldithiocarbamic acid (DEDTC), 8-hydroxyquinoline (oxine), α-benzoinoxime (cupron), N-nitrosophenylhydroxylamine (cupferron), diazoaminobenzene, p-aminophenol, o-nitrophenol, 1–3, 4-trihydroxychalcone, p, p'-methylene bis N,N-dimethylaniline and 2-hydroxy-3-methoxybenzaldehyde. Only the first four of these were effective in

[2] I am indebted to the Bersworth Chemical Company, Framingham, Massachusetts, for samples of this substance; and to Dr. Linus Pauling for suggesting its use in connection with these experiments.

extending the life-span of the sperm. However, since the others were each tested in only one set of experiments there is the possibility that some of these might prove effective on further investigation.

The results of experiments with the first four substances are summarized in Table II. Of these, Versene and DEDTC have given the longest extensions of life-span. Versene proved to be highly effective at concentrations from 10^{-3} to 10^{-5} molar and DEDTC at concentrations of 10^{-3} and 10^{-4}. Two experiments not listed in the table also show DEDTC to be effective at 10^{-5} molar (7 hours half-motile life vs. 1½ hours for the sea water control with 1% Lytechinus sperm). Both substances were inhibitory at 10^{-2} molar.

TABLE II

Action of various metal-chelating agents on life-span of sea urchin spermatozoa at 19°–22° C.

Substance	Species	Semen conc.	No. of experiments	Half-motile life* and half-fertilizable life† in: (concentration in sea water)						
				0	10^{-3} *M*	10^{-4} *M*	10^{-5} *M*	10^{-6} *M*	10^{-6} *M*	10^{-7} *M*
				hrs.	hrs.	hrs.	hrs.	hrs.	hrs.	hrs.
Versene[1]	*S. purp.*	1%	8*	2.9	0.3	21	22	13	7	7
	S. purp.	1%	3†	1	<1	20+	20+	10–15		
	S. purp.	0.1%	4*	0.2	<0.1	3	10	1.5	0.3	0.2
	L. pict.	1%	4*	3.2		21	27	29		
	L. pict.	1%	2†	0.8		24+	24+	24+		
	L. pict.	0.1%	1*	0.2		18	22	13		
DEDTC[2]	*S. purp.*	1%	3*	5.6	2.5	17	5.5			
	L. pict.	1%	8*	5.8	2.7	22	32			
	L. pict.	1%	2†	1			30+			
	L. pict.	0.1%	1*	<0.5	1	4	2.5			
Oxine[3]	*S. purp.*	1%	3*	2		16	14	2		
	S. purp.	0.1%	1*	0.1		2	2	0.1		
	L. pict.	1%	2*	0.4		4.5		1.5	0.8	0.5
Cupron[4]	*S. purp.*	1%	2*	1			6	8.5	5	
	S. purp.	0.1%	2*	0.3			2	2	0.3	

[1] Ethylenediaminetetraacetic acid.
[2] Diethyldithiocarbamate.
[3] 8-Hydroxyquinoline.
[4] α-Benzoinoxime.

The degree of extension of life-span obtained with these agents is of the same order as was obtained by use of the amino acids. It seems reasonable to conclude, then, that they are acting in similar manner, namely by virtue of their metal-chelating capacity. Differences in effective range of concentrations can be attributed to differences in the dissociation constants of the metal-chelate compounds. Martell and Calvin (1952) have assembled data on the stability constants (reciprocal of dissociation constants) for an extensive series of metal-chelate compounds, including most of the agents used in this work. While the data on optimum concentrations accumulated here do not permit detailed quantitative comparisons, qualitatively the differences between the amino acid glycine and the other chelating agents, such as Versene, correspond to the differences in their relative avidity for metal ions.

Life-span of spermatozoa in artificial sea water with various amounts of calcium. In order to examine further the view that the extension of life-span by the amino acids and other metal-chelating agents was occasioned by the binding of certain metal ions, tests were made with artificial sea water. This was prepared from Merck Reagent Grade chemicals, of which the NaCl was especially low ($< 0.0001\%$) in heavy metals. Based on the sea water analyses of Lyman and Fleming (1940) the artificial sea water was made up with the following composition: 1000 ml. 0.55 *M* NaCl, 22 ml. 0.55 *M* KCl, 195 ml. 0.37 *M* $MgCl_2$, 103 ml. 0.37 *M* Na_2SO_4, 6 ml. 0.55 *M* $NaHCO_3$ and 35 ml. 0.37 *M* $CaCl_2$, adjusted to pH 8.2.

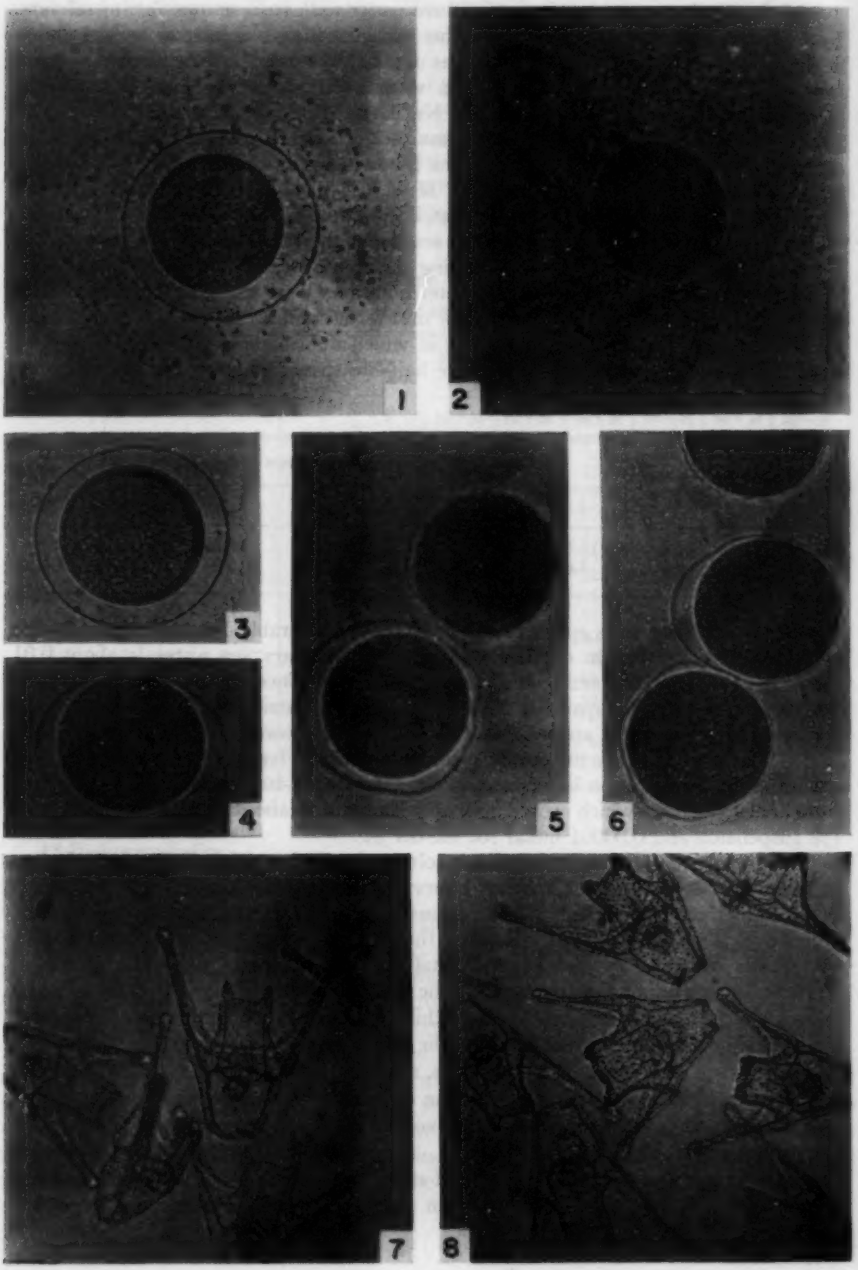
Along with the tests of the artificial sea water the effect of varying the concentration of calcium was also examined, using the above formula with various amounts of the $CaCl_2$ solution. Eight sets of determinations were made of the duration of motility of sperm of Strongylocentrotus in 1% and 0.1% suspension in ordinary sea water and in the artificial sea waters in which the calcium concentration ranged from 0 to 8×10^{-2} molar. The average half-life-spans, in hours, in these experiments were:

	Ordinary sea water	Artificial sea water containing calcium at molar concentration						
		0	0.0006	0.0025	0.01	0.02	0.04	0.08
1% sperm	3.7	15.1	14.6	15.3	16.1	11.3	12.4	8.2
0.1% sperm	0.7	3.6	4.1	3.3	6.7	0.8	1.6	1.4

As the figures show, the spermatozoa survived considerably longer in most of the artificial solutions than in ordinary sea water. Ordinary sea water is about 0.01 molar in calcium. The artificial sea water with this concentration of calcium gave the longest average life-span for the 0.1% sperm suspension but with the 1% suspension it did not differ appreciably from those with lower concentrations. Even in the "Ca-free" solution the sperm were found to survive longer than in ordinary sea water. This solution is, of course, not actually Ca-free since the added semen contributes calcium, which would probably amount to about 0.0001 molar for the 1% suspension and 0.00001 molar for the 0.1%.

It appears, then, that a balanced salt solution made from chemicals relatively low in heavy metals enables the sperm to survive longer than does natural sea water. Since this effect is obtained with solutions in which the concentration of calcium varies over a very wide range it is clear that this ion is not primarily concerned in the results obtained by use of the metal-chelating agents. Thus the action of Versene in extending the life-span of the sperm is not simply attributable to its known ability to bind calcium. While this chelating agent forms complexes with other alkaline earth metals the fact that the amino acids such as glycine, which form relatively weak complexes with these ions, are effective would tend to rule them out as being primarily involved in the life-span extending effect. It seems most reasonable to conclude that heavy metals are involved, although the present evidence does not permit ready identification of these. Rothschild and Tuft (1950) found that the dilution effect of sea water on rate of oxygen uptake could be imitated by trace amounts of $CuCl_2$ or $ZnCl_2$ dissolved in isotonic 'Analar' (containing negligible

PLATE I. Eggs and embryos of *Lytechinus pictus*. Magnifications: 175 × for Figures 1 to 6 and 65 × for Figures 7–8.



amounts of heavy metal) NaCl. The isotonic 'Analar' NaCl itself does not give a dilution effect when added to the sperm suspension. Glycine solutions can also abolish the dilution effect (Tyler and Rothschild, 1951). On the basis of this evidence it is suggested that Cu^{++} and Zn^{++} are among the heavy metal ions whose removal is responsible for the life-span extending effect of the chelating agents.

Effect of treatment of the sperm on membrane-elevation. As was mentioned earlier, treatment of the sperm with solutions of the amino acids improves the fertilization-reaction induced upon insemination with dilute and with aged sperm suspensions. The same effect is obtained with other chelating agents. In Plate I examples of good and poor membrane-elevation of eggs of Lytechinus are shown. The effect on the fertilization-reaction is illustrated by the results of the experiments with glycine and with Versene presented in Table 3. It may be noted that with sperm diluted in ordinary sea water, and aged only a short time, the type of reaction, along with the percentage fertilization, improves upon insemination with increasing amounts of sperm. However, with sperm that have been diluted in glycine or in Versene, and similarly aged, much smaller amounts suffice to give 100 per cent fertilization and normal membrane-elevation. Upon aging, the rapid drop-off in fertilizing capacity is seen along with the poor fertilization-reaction induced by the sea water-sperm. The glycine- or Versene-treated sperm, upon aging, maintain not only their fertilizing capacity but also their ability to induce good membrane-elevation. Towards the end of their extended life-span they, too, may induce poor membrane-elevation when used in amounts that were earlier effective in inducing a good reaction and 100 per cent fertilization. The type of reaction given by the eggs is evidently dependent upon the amount of sperm used for insemination as well as the age of the suspension. This holds both for the sea water-sperm and for the glycine- or Versene-treated sperm. Excessive amounts of sperm may sometimes be inhibitory, as the figures in Table VII indicate. Occasionally, also, sea water-sperm fail to give good membrane-elevation with any amount tested while the glycine- or Versene-treated sperm induce normal membrane-elevation.

It appears from these results that the condition of the inseminating sperm can determine the type of fertilization-reaction given by the eggs. As the spermatozoa age in ordinary sea water an increasing proportion of them become impaired in such a way that, while still capable of fertilization, they cannot elicit a normal response on the part of the egg. The relatively poorer response elicited by the smaller

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FIGURE 1. Good membrane-elevation shown by egg with intact jelly-coat inseminated with sperm treated with Versene (10^{-3} molar in sea-water, ½ hour).

FIGURE 2. Poor membrane-elevation shown by egg with intact jelly-coat inseminated with about 8 times as much sea water-treated sperm (note larger number of sperm in the jelly-coat than in that of Figure 1).

FIGURE 3. Good membrane-elevation shown by jellyless egg inseminated with Versene (10^{-4} *M*)-treated sperm.

FIGURE 4. Poor membrane-elevation shown by jellyless egg inseminated with very small amount of Versene (10^{-4} *M*)-treated sperm.

FIGURES 5 AND 6. Various types of poor membrane-elevation shown by Versene (10^{-3} *M*)-treated and sea water-washed eggs inseminated with sea water-treated sperm.

FIGURES 7 AND 8. Five day old plutei (not fed) from eggs fertilized and allowed to develop in sea water (Fig. 7) and in 10^{-3} molar Versene in sea water (Fig. 8).

amounts of inseminate can be interpreted on the basis of their greater dilution in the insemination dishes and consequent increase in proportion of impaired sperm in the interval before fertilization. The poor response given on occasion with excess sea water-sperm can be attributed to an increased probability of the impaired sperm, present in high proportion in such suspension, encountering the eggs; whereas with somewhat lower amounts of inseminate the more motile and presumably unimpaired sperm would have the advantage. Whether or not other factors, such as the liberation of antifertilizin from the sperm (see Tyler and Atkinson, 1950), might also be involved, would need to be further investigated.

TABLE III

Fertilization-reaction of eggs of Lytechinus inseminated with various amounts of sperm that have aged in sea water (s.w.), 0.05 M glycine (gl) and 0.001 M Versene (ve)

Age of 1% sperm susp.	Solution	Percentage of good membrane-elevation (G) and poor membrane-elevation (P) upon insemination of ca. 500 eggs in 4 ml. of sea water with following volumes of 1% sperm aged as indicated							
		0.05 ml.		0.10 ml.		0.2 ml.		0.4 ml.	
		G	P	G	P	G	P	G	P
(hours)									
0.02	s.w.	10	80	25	75	100	0	100	0
	gl.	100	0	100	0	100	0	100	0
0.5	s.w.	1	20	0	20	10	75	95	5
	gl.	100	0	98	0	100	0	100	0
1, 2, 5 and 10	s.w.	0	0	0	0	0	0	0	0
	gl.	100	0	100	0	100	0	100	0
18	s.w.	0	0	0	0	0	0	0	0
	gl.	90	5	100	0	95	5	100	0
23	s.w.	0	0	0	0	0	0	0	0
	gl.	0	15	10	30	35	50	85	15
0.2	s.w.	10	75	90	10	100	0	100	0
	ve.	100	0	100	0	90	10	100	10
1	s.w.	0.2	0	10	20	20	60	90	10
	ve.	100	0	100	0	100	0	100	0
2 and 4	s.w.	0	0	0	0	0	0	0	0
	ve.	100	0	100	0	100	0	100	0
10	s.w.	0	0	0	0	0	0	0	0
	ve.	15	3	95	5	100	0	100	0
21	s.w.	0	0	0	0	0	0	0	0
	ve.	30	5	45	10	85	5	100	0

Fertilization-reaction of Versene-treated eggs in Versene and in sea water. As was noted above (see introduction), treatment of sea urchin eggs with glycine, and fertilization in the solution, improves their fertilizability and type of reaction. This occurs also with other chelating agents. Table IV gives the results of some experiments with Versene. Comparison of the last three lines of the table with the first line shows the great improvement in percentage fertilization and in membrane-elevation that is obtained upon insemination in the Versene solutions. The sperm used in this experiment were freshly diluted in sea water but, of course,

TABLE IV

Fertilization-reaction of Versene-treated eggs of Lytechinus inseminated in sea water and in Versene

Eggs treated for ½ hour with	0.1 ml. of egg-suspension transferred to 4 ml. of	Percentage good membranes (G) and poor membranes (P) upon insemination with following volumes of 1% sperm			
		0.05 ml.		0.20 ml.	
		G	P	G	P
Sea water	Sea water	0	30	5	75
10^{-6} *M* Versene	Sea water	70	30	80	20
10^{-4} *M* Versene	Sea water	60	40	100	0
10^{-3} *M* Versene	Sea water	100	0	100	0
10^{-6} *M* Versene	10^{-6} *M* Versene	100	0	100	0
10^{-4} *M* Versene	10^{-4} *M* Versene	100	0	100	0
10^{-3} *M* Versene	10^{-3} *M* Versene	100	0	100	0

upon insemination of the eggs in the Versene solutions they are exposed to the action of this agent. So this type of experiment does not permit one to decide whether the effect is on the egg or sperm or both.

If the eggs are inseminated in sea water after treatment with Versene (lines 2, 3, and 4 of Table IV) there is much less improvement in the fertilization-reaction. In these tests insemination was done immediately after transfer to the sea water, and without further washing. It seemed likely that there might be sufficient carry-over of the Versene, which would perhaps diffuse only slowly from the gelatinous coat of the egg, to affect the results. Tests were therefore made of the effect of washing the eggs, after Versene-treatment, by repeated transfer in sea water or in Versene. The results of such a test are given in Table V. As the figures show, successive washing in sea water decreases the fertilizability of the Versene-treated eggs while those correspondingly transferred through Versene solution maintain their high fertilizability and ability to elevate good membranes.

Effect of removal of jelly-coat. It has been observed that the jelly-coat of eggs of various sea urchins swells considerably in solutions of amino acids and of proteins (Runnström *et al.*, 1943, 1946). This effect is readily observed, too, in Versene and DEDTC solutions. Thus, in 10^{-3} *M* Versene the jelly-coat of Lytechinus eggs swells within a half-hour to about double its original thickness.

TABLE V

Effect of washing in sea water on fertilization-reaction of Versene-treated eggs of Lytechinus

Eggs treated 2 hrs. in 10^{-3} *M* Versene and washed in:	Percentage good membranes (G) and poor membranes (P) upon insemination with 0.05 ml. of 1% sperm. Eggs washed by transferring 0.1 ml. to 4 ml. of new solution							
	First washing		Second washing		Third washing		Fourth washing	
	G	P	G	P	G	P	G	P
Sea water	25	75	0	50	0	30	0	5
10^{-3} *M* Versene	100	0	100	0	100	0	100	0

Concerning the action of the amino acids and proteins on the egg Runnström (1949, p. 251) points out that "This may facilitate penetration of spermatozoa and elevation of the fertilization membrane. But this is not the whole explanation of their improving effect." Eggs deprived of their jelly-coat when inseminated in these solutions also showed improved fertilization and membrane-elevation (*cf.*, Wicklund and Gustafson, 1949). It is suggested (Runnström, 1948) that these substances overcome a "cytoplasmic underripeness" of the egg. As the present work shows and was earlier reported (Tyler and Atkinson, 1950), treatment of the sperm alone with glycine, Versene, etc. induces good membrane-elevation. Treatment of the sperm with albumin (Wicklund, 1949) also has this effect. Also, increasing the amount of sea water-sperm used for insemination can result in improved membrane-elevation. It is not easy to see how a "cytoplasmic underripeness" can be involved in this.

In order to examine these questions further the reaction of jellyless eggs was investigated. The results of an experiment with Lytechinus eggs are given in

TABLE VI

Effect of removal of jelly-coat on fertilization-reaction of eggs of Lytechinus

Volume of freshly diluted 1% sperm used for insemination	Percentage good membranes (G) and poor membranes (P) upon insemination in sea water after similar washing in sea water:					
	I. Eggs from sea water suspension with intact jelly-coat		II. Eggs from (I) shaken to remove jelly-coat		III. Eggs treated ½ hour in 10^{-2} *M* Versene, shaken to remove jelly-coat	
	G	P	G	P	G	P
0.4 ml.	100	0	90	10	100	0
0.2 ml.	100	0	60	30	60	40
0.1 ml.	90	10	20	20	10	50
0.05 ml.	10	80	10	5	10	15
0.025 ml.	5	45	0.5	0.5	3	2
0.013 ml.	0	5	0.2	0.2	1	0.5

Table VI. The eggs had stood for ½ hour in 10^{-2} molar Versene (III) and in sea water (II) and were then shaken to remove their jelly-coat. That the jelly-coat was effectively removed was checked by microscopic examination. They were then washed in several changes of sea water and aliquots inseminated with various amounts of a freshly diluted sea water-sperm suspension along with aliquots of similarly washed sea water-eggs with intact jelly-coat (I). Comparison of II with III in this table shows that the pretreatment with Versene occasioned no significant improvement in the fertilization-reaction or in the percentage fertilization obtained with a given amount of sperm. The eggs with intact jelly-coat (I) showed better fertilization with corresponding amounts of sperm, and this is consistent with earlier experiments (Tyler, 1941; Runnström, 1947) along this line. Similar results have been obtained with eggs pretreated with glycine and inseminated in sea water after removal of the jelly-coat. Also pretreatment of jellyless eggs with glycine or with Versene and insemination in sea water did not improve fertilization in comparison with the sea water-exposed jellyless eggs.

Since the action of these agents does not persist when the eggs are returned to sea water it does not seem likely that a "cytoplasmic maturation," in whatever sense Runnström (1948) may mean this, can account for the results. On the other hand treatment of the sperm and insemination of jellyless eggs in sea water results in improved fertilization, as it does in eggs with intact jelly-coat (Table III). Three such experiments are listed in Table VII. The eggs were pretreated with Versene in order to facilitate removal of the jelly-coat, washed and inseminated in sea water with various amounts of sperm that had been diluted and aged briefly in 10^{-3} *M* Versene or in sea water. Comparison of the Versene-treated with the sea water-sperm shows the former to have some 8 to 16 times the fertilizing capacity of the latter, and to induce good membrane-elevation when amounts of sperm are

TABLE VII

Fertilization-reaction of jellyless eggs of Lytechinus inseminated with various amounts of Versene-treated and untreated sperm

Volume of 1% sperm used for insemination	Percentage good membranes (G) and poor membranes (P). Eggs treated for ½ to 1 hour in 10^{-3} *M* Versene, shaken to remove jelly-coat, transferred to sea water (4 ml.) and inseminated with indicated volumes of sperm at 10 to 15 minutes after dilution in:											
	(Experiment I)				(Experiment II)				(Experiment III)			
	Sea water		10^{-3} *M* Vers.		Sea water		10^{-3} *M* Vers.		Sea water		10^{-3} *M* Vers.	
	G	P	G	P	G	P	G	P	G	P	G	P
ml.												
0.8	80	20	100	0	0	100	100	0	0	100	100	0
0.4	100	0	100	0	20	80	100	0	5	95	100	0
0.2	95	5	90	10	60	30	80	20	60	40	100	0
0.1	40	60	99	1	20	20	100	0	10	50	40	60
0.05	5	90	97	3	10	5	100	0	10	15	90	10
0.025	5	15	99	1	0.5	0.5	98	2	3	2	60	40
0.0125	2	3	97	3	0.2	0.2	45	50	1	0.5	10	85
0.0063	0	0	20	40	0	0	20	5	0	0	30	20
0.0031	0	0	20	10	0	0	45	5	0	0	20	5
0.0016	0	0	8	2								
0.0008	0	0	0	0								

used that, in the case of the sea water controls, give mostly a poor fertilization-reaction. In both, the effect of quantity of sperm on type of reaction is shown, but with the Versene-treated sperm good membrane-elevation is given by considerably less sperm. This was illustrated also in the experiments on eggs with intact jelly-coat (Table III). An additional feature is observed in the results of the experiments of Table VII. With large amounts of sea water-sperm (0.8 ml. for experiment I; 0.8 and 0.4 ml. for experiments II and III) the fertilization-reaction is poorer than with the next lower amounts. This "optimum sperm concentration" effect is not regularly encountered in other experiments and presumably may be a property of particular sperm suspensions. An interpretation of this is offered in a preceding section. It may be concluded from the experiments with jellyless eggs that the improved fertilization-reaction induced in eggs with intact jelly-coat by

treated sperm is not primarily due to an effect of such sperm on the jelly-coat or to greater ease of penetration of the jelly-coat.

Development in glycine and in Versene solutions. Glycine, in rather low concentration, interferes with development. Thus in a test with Lytechinus eggs, fertilized and allowed to remain in 10^{-3} molar glycine, development did not proceed beyond the formation of abnormal gastrulae. In stronger solutions stereoblastulae were generally formed. While fertilization and cleavage are obtained in solutions as strong as 0.125 molar, development generally stops in the early blastula and disintegration soon sets in. Transfer to sea water soon after fertilization permits normal development, as Wicklund and Gustafson (1949) found with weaker solutions. In Versene solutions development of Lytechinus eggs was found to proceed quite normally in concentrations up to 0.001 molar. In one test, for example, eggs inseminated in 10^{-3}, 10^{-4} and 10^{-5} molar Versene gave 100 per cent fertilization and practically all eggs developed normally to the pluteus stage. Five-day old plutei from the 10^{-3} molar Versene solution and the sea water control are shown in Figures 7 and 8. Also eggs of Lychechinus that have been fertilized by sperm that have aged up to 24 hours in 10^{-3} molar Versene were found to develop normally to the pluteus stage.

Discussion

Most of the points raised by the present experiments have already been discussed above, but a few may be further emphasized here.

The ability of the metal-chelating agents, Versene, DEDTC, oxine and cupron, and of artificial sea water of low metal content, to extend the life-span of spermatozoa provides strong support for the previously expressed view (Tyler and Rothschild, 1951) that the trace metals normally present in sea water are responsible for the usual early death of the sperm in this medium. The life-span extending action of the amino acids and peptides (Tyler, 1950; Tyler and Atkinson, 1950) is explainable on this basis as is also the action of protein (Metz, 1945; Wicklund, 1949) and seminal fluid (Hayashi, 1945). In birds and mammals, amino acids, protein and seminal fluid may be similarly involved (Lorenz and Tyler, 1951; Tyler and Tanabe, 1952; Chang, 1947, 1949).

Very likely seminal fluid owes its action largely to its proteins. This can help explain the well-known *Dilution Effect; i.e.,* the decrease in life span with increasing dilution of the semen in sea water or physiological salt solutions. Thus, in the denser suspensions more seminal fluid protein would be available to bind the heavy metals present and eliminate their toxic action. If this were the whole explanation of the dilution effect then in the presence of the proper concentration of metal-chelating agent the dilute suspensions should survive as long as the denser ones. While this has been approached in some experiments reported here, in most cases the dilute suspensions have not lasted as long. So, the question remains open as to what extent other factors (see Gray, 1928a, 1928b, 1931; Rothschild, 1951) may be involved. However, it is now clear (*cf.,* Tyler and Rothschild, 1951) that the early death of sperm diluted in ordinary sea water is not due to exhaustion of endogenous food reserves.

Identification of the particular heavy metals that may be involved in the toxic action of ordinary sea water on sperm is not readily feasible from the present re-

sults. As mentioned above there are good reasons for suspecting Cu^{++} and Zn^{++}, but it is quite possible that others may also be concerned.

Of special interest is the ability of spermatozoa treated with amino acids and other chelating agents to improve the fertilization-reaction of the eggs. It is evident that the type of response given by the egg is not simply dependent upon the condition of the egg itself. The spermatozoon does not act in all-or-none manner in the sense of operating, or failing to operate, a trigger mechanism. Dependent upon its own condition the spermatozoon can elicit good or poor membrane-elevation on the part of the egg. This is not simply a matter of the treated sperm being able to traverse, and perhaps soften, the jelly-coat of the egg more effectively, since the effect is manifest also with jellyless eggs (Table VII).

In regard to possible effects on the egg the present experiments tend to rule out any maturing action. While insemination in Versene or glycine solutions improves fertilization, it is clear that this can be largely due to the effect on the sperm. Since pretreatment of the eggs with Versene or glycine and subsequent washing, and insemination in sea water, give no marked improvement in fertilization it appears that if there is any effect on the egg it is readily reversed. These substances cannot be considered to overcome any supposed "cytoplasmic underripeness" (Runnström, 1948, 1949) of the egg.[3] There are evidently effects of these substances on the eggs as manifest by swelling of the jelly coat and apparently also changes in the egg cytoplasm or its surface (*cf.*, Runnström and Monné, 1945; Runnström, 1948). However if these changes have any action in the direction of improved fertilizability of the egg it is evident that such effect largely disappears upon return to ordinary sea water, and it could hardly be considered a "maturating" effect. One might attempt to assess a possible fertilization-favoring effect on the egg by comparison of the results of inseminating also with treated-sperm eggs in sea water and in a solution of chelating agent. However, it would be difficult to decide to what extent the results are influenced by the effect on the sperm of the two different media in the insemination dishes. Runnström and his coworkers have contributed much to our knowledge of fertilization. While their extensive studies on "underripe" and "overripe" eggs do not represent a major part of their contributions it would appear desirable to re-examine the application of these terms to eggs. When difficulties in fertilization are encountered it would be important in the first place to know to what extent this is due to some agent ordinarily present in the sea water. Certainly where treatment of the sperm alone can improve the fertilization-reaction it does not seem reasonable to assume an "underripe" or "overripe" condition of the egg.

I am greatly indebted to Dr. T. Y. Tanabe, now at the Pennsylvania State College, for his help in this work, and also to Mrs. Joan Merritt for her participation in some of the later phases.

[3] While this manuscript was in preparation Dr. Hans Borei sent the author a copy of a note which he had prepared for publication in Experimental Cell Research. He reports that insemination in Versene solutions improves the fertilizability of underripe and overripe eggs of *Psammechinus miliaris*. Since pretreatment and similar tests were not performed he is undecided as to whether the increased fertilizability is due to effects on the eggs, the sperm, or both.

Summary

1. The duration of motility and of fertilizing capacity of sea urchin sperm diluted in sea water can be considerably extended by the addition of any one of certain metal-chelating agents. These include ethylenediaminetetraacetate (Versene), diethyldithiocarbamate, 8-hydroxyquinoline, α-benzoinoxime and various amino acids and peptides (glycine, alanine, valine, leucine, lysine, glutamic acid, histidine, phenylalanine, tryptophane, cysteine, glycylglycine, glutathione). The relative increase in life-span, in the presence of these agents, is greater the more dilute the sperm suspension, while the absolute increase is generally greater for the more concentrated suspensions. Over 100-fold extension of life-span of dilute suspensions has been obtained by use of these agents.

2. An artificial sea water of low heavy metal content also enables the sperm to survive longer than in ordinary sea water. This effect is obtained with artificial solutions in which the calcium concentration ranges from 8 times to 1/100 that of sea water.

3. The results support the previously suggested view that the increased survival of the sperm in presence of amino acids, proteins, etc. is due to the ability of these agents to bind heavy metals present in the dilution medium. It is suggested that Cu^{++} and Zn^{++} are among the metals involved.

4. The "Dilution Effect" (decreasing life-span with increasing dilution of suspension) is largely explained on the basis of similar action of the seminal fluid proteins which are present in higher concentration in the denser suspensions prepared by direct dilution of the semen.

5. Treatment of the sperm with glycine or with Versene improves the fertilization-reaction (membrane-elevation) induced upon insemination of eggs in sea water with a given amount and age of sperm suspension. This effect persists along with the increased survival of the sperm in these agents. The results demonstrate that the type of response given by the egg can be determined by the condition of the inseminating sperm. The spermatozoon evidently does not simply act in "all-or-none" manner, in the sense of operating a trigger mechanism in the egg.

6. Fertilization is also improved when the eggs are inseminated in the presence of Versene or glycine, but the effect is largely eliminated if the eggs are washed and inseminated in sea water. Swelling of the jelly-coat that occurs in these solutions is not an important factor in the results. Eggs that have been deprived of their jelly-coat behave similarly, showing improved fertilization in the solutions and no persistent pretreatment effect. Such eggs also show the improved response to treated sperm. It is concluded that if there is any effect of these agents on the egg in the direction of improving fertilizability such effect is readily reversed upon transfer to sea water and does not constitute a "maturating" effect.

7. Eggs of *L. pictus* and *S. purpuratus* develop normally to the pluteus stage in 10^{-3} molar Versene but not in 10^{-3} molar glycine.

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SOME PHYSIOLOGICAL ASPECTS OF REPRODUCTION IN XIPHOPHORUS MACULATUS

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Live-bearing fishes are being used extensively in biological research and instruction in genetics, embryology, endocrinology, pigmentation studies, and other fields to which these fishes can readily be adapted. Turner (1937) has carefully worked out the reproductive cycle of *Xiphophorus* from the viewpoint of ova development, period of fertilization, and superfetation. The present study is concerned with the reproductive cycle in this fish from the viewpoint of spermatozoa viability, selection and competition and the duration of ova production. Experiments have been conducted to show the number of young that can be born during the reproductive activity of the female and to show the duration of life of spermatozoa within the female genital tract and the competition and selection involved.

Animals and Their Treatment

The fishes used in this experiment were kept in aquaria 20 × 25 × 20 cm., which had a layer of sand on the bottom about two inches deep. Each aquarium contained ample amounts of *Vallisneria* or *Sagitarria* planted along the back wall and two sides, allowing full observation from the front. Young fishes were kept in larger aquaria measuring 30 × 26 × 26 cm. until almost sexually mature; then they were segregated according to sex in the smaller type of aquarium. Prior to giving birth to young, gravid females were placed in aquaria of the smaller type heavily planted with hornwort (*Ceratophyllum*). After the young were born, the females were removed to other aquaria of the same size. The young were counted in the late afternoon of the day they were born and then returned to the same aquaria. No more than three sexually mature fish were kept in one aquarium.

The diet for mature fish throughout the experiment consisted of white worms, mosquito larvae, Daphnia, cyclops, prepared dry food and a dried and ground mixture of strained liver, spinach and bran flakes. Young fish were fed "micro worms" (Nematoda), prepared dry food, Daphnia and white worms. Mature fish were fed once a day. Young fish were fed three or four times daily.

Two kinds of water were used. More commonly the water was taken directly from the tap and allowed to stand in earthenware crocks or porcelain-lined tubs for about two weeks before being used. At other times water was used that had been filtered through charcoal. This was allowed to remain about a week in the tubs before being used. No adverse effect of the water used was noted during the

[1] The writer wishes to thank Dr. W. C. Allee of the University of Florida for the help and kind supervision that he has given to this work. He also wishes to thank Dr. A. W. Bellamy of the University of California and Dr. Myron Gordon of the American Museum of Natural History for their generosity in supplying the fish that were used in these experiments.

course of the experiments. A routine schedule was maintained for cleaning the aquaria whereby each aquarium was thoroughly cleaned once each month.

The fish were kept in a greenhouse which has crude temperature control. In the winter months the temperature averaged about 75° F. during the day. During the last week of February, however, the steam supply was drastically reduced, and the temperatures were somewhat lower. During the summer months the control was much less perfect.

During the eight-months period that the fishes were kept under these conditions only three fishes died. Two of these, males of unknown age, slowly wasted away; the other, a female, died suddenly a few days before she should have given birth to her first brood of young. The reasons for these deaths could not be determined.

The species used in these experiments were *Xiphophorus maculatus, Xiphophorus variatus* and *Xiphophorus helleri—Xiphophorus maculatus* hybrids. Various color forms of *X. maculatus* were used. These included forms without any peduncular or caudal pattern, the one-spot pattern, the crescent pattern, the wag pattern, and the stippled, spotted, red, blue and red-bellied color forms. Matings were usually made between color forms and identity of young based upon color patterns.

Experiments and Results

The experiments were of two types. In one experiment (Group I) female fish were isolated from contact with any male fish and records kept of the number of broods of young produced by them during the period of isolation. In some cases females were used which had been kept in aquaria for some time with males of their own species (Group I-A). Whether these females had given birth prior to being isolated cannot accurately be determined; therefore, the number of broods produced by these females is not considered as maximum. In another group (Group I-B) virgin females were used. After fertilization was accomplished these fish were isolated as in the previous experiment.

Another group of fish was kept in contact with males of their own species but of different color form and males of different species (Group II). In some cases the contact consisted only of the brief period of courtship and insemination (Group II-A). In other cases, (Group II-B), the contact with the male was constant except for the time that the female was removed for a few days prior to giving birth to a brood of young. (The term brood is used as defined by Turner (1937) to indicate a group of growing and differentiating ovocytes of approximately equal development up to the time of fertilization and also the embryos produced by the fertilization of these ova up to the time of birth.)

In some cases copulation was observed, but no young were produced by these females. These cases will be considered as Group III.

Group I-A

A group of five females was kept isolated in small aquaria throughout the experiment. Records were kept of the number of broods, the number of young produced by these females and the interval of time between broods. These females had already been inseminated when the experiments began, and it is not known

whether broods were produced by the females prior to this time. Therefore, the number of broods produced by these females cannot be considered as maximum. The results of this experiment are recorded in Table I. These results show a fairly consistent trend; that is, the number of young in the last brood is smaller than the usual number of young in the broods of each female. Fishes 0–1, 0–2, 0–4 and 0–5 were kept in contact with males after it was believed that they had produced their last broods.

At autopsy these fish showed the following results:

0–1. Fifty-three days after delivery of the fifth brood of one embryo and after 18 days of contact with male B-20, autopsy showed 16 large, deep amber ova; two large, light-colored ova; 16 embryos well developed; one embryo only slightly developed; and many small, white ova in the ovary.

0–2. Fifty-three days after delivery of the fifth brood of one embryo and after 18 days of contact with male M-10, autopsy showed 23 large, deep amber ova; two embryos about one-half developed; and many small, white ova in the ovary.

TABLE I

Reproduction records of females of Group I-A

Females	Number in Brood A	Interval	Number in Brood B	Interval	Number in Brood C	Interval	Number in Brood D	Interval	Number in Brood E
0–1	8	37	30	35	30	31	33	37	1
0–2	8	36	24	33	26	36	8	30	1
0–3*	19	30	9	35	8	30	9		
0–4	16	36	7	31	1				
0–5	8	35	6						
0–6	7	32	26	32	23	33	4		

* Female 0–3 was kept in constant contact with male 0–20.

0–4. Ninety-eight days after delivery of the fourth brood and after 20 days of contact with male M-11, autopsy showed about 40 large, deep amber ova; several small, white ova; but no embryos.

0–5. One hundred and thirty-one days after delivery of the second brood and after 20 days of contact with male M-12, autopsy showed 34 embryos about three-quarters developed; several small, white ova; but no large, deep amber ova.

The mean interval between broods for these fish was 33½ days.

It seems justified to assume that these females ceased to produce young because of lack of viable spermatozoa within the ovary. With the possible exception of 0–4, none had reached the end of the reproductive period. Whether successful insemination was accomplished with female 0–4 is, of course, open to question. Copulation was observed, but it does not necessarily follow that the female was inseminated.

Group I-B

In seven matings the females gave birth to a limited number of broods. They were kept isolated in small, heavily planted aquaria and examined every day for

TABLE II

Reproduction records of females of Group I-B

Mating		Number of Broods	Total number of young	Time in days*	Days from last brood to autopsy
Female	Male				
C-1	C-20	4	56	134	34
C-5	R-10	3	58	103	29
C-7	K-20	3	56	101	33
K-2	K-20	3	48	97	73
K-3	B-20	3	60	104	66
K-6	C-15	4	79	132	40
N-3	P-20	3	71	102	66

* From insemination to birth of last brood.

young. At the end of the experiment these females were autopsied and the condition of the ovary noted. These data are recorded in Table II. Evidently, the viable spermatozoa that were introduced at the insemination were depleted. Although the females were capable of producing more broods, none were produced because the lack of viable spermatozoa in the ovary or genital tract made fertilization impossible.. Popular literature about aquarium fishes sets the upper limit for the number of broods produced in isolation at eight broods. This limit was not reached in these experiments. In all instances autopsy revealed that the ovary contained large, deep amber ova; small, white ova; but no embryos.

TABLE III

Reproduction records of females of Group II-A

Female	Insemination date	Interval to Brood A	Interval to Brood B	Interval to Brood C	Interval to Brood D	Interval to Brood E
C-1	II-6	38	32	32	33	
C-2	VI-6	48				
C-3	VI-7	45				
C-4	VI-12	42				
C-5	III-15	41	31	31		
C-6	V-15	42	29			
C-7	III-13	40	31	30		
C-8	IV-26	50	31			
Ca-2	II-11	40	32	30	32	28
Ck-1	VI-6	37				
K-1	II-6	57	31	31	27	
K-2	II-4	39	26	32		
K-3	II-4	42	32	30		
K-5	V-31	44				
K-6	II-4	40	32	28	32	
N-2	IV-26	42	30			
N-3	II-6	39	31	33		
P-3	VI-5	31				
P-5	V-18	35				

Group II-A

Soon after the experiments were begun it was noted that the virgin females usually did not give birth to their first brood until about 40 days after fertilization. Since copulation had been observed in each case and the female isolated from all contacts immediately after insemination, accurate dating of the period between insemination and birth of the brood was possible. Table III shows the periods in days between insemination and birth of first brood.

This table shows that for the cases listed the average period between insemination and birth of first brood was 41.7 days. When this period is compared with the periods between subsequent broods for these females, it is evident that some factor is involved which delays the birth of the first brood. What this factor is was not ascertained in this study.

That it is due to some delayed action on the part of spermatozoa is not plausible in the light of one experiment in which a female was inseminated by two males at different times. Forty-two days after insemination by the first male the female gave birth to her first brood of young. On that day the female was fertilized by the second male. Thirty days after this second insemination the second brood was born. Of the 41 young, 28 were of the same phenotype as the second male. In this case the spermatozoa fertilized ova that developed in the normal thirty-day period.

Group II-B

Several attempts were made to establish the degree of competition between the spermatozoa of different males. In some cases females were used which had been inseminated by males of their own species or hybrid type. After giving birth to broods of young, these females were placed in constant contact with males of different species or males of the same species but of a phenotype different from the original male. The results of these experiments are as follows:

1. A *X. maculatus* female, K-1, was inseminated by a male *X. variatus,* V-20. Four days after the birth of the second brood from this mating (a period of 92 days from insemination) the female was placed in constant contact with a male *X. maculatus*. During the contact with this male, two broods of young were born. Although the males differed phenotypically and the young produced from ova fertilized by spermatozoa of the *X. maculatus* male could have readily been distinguished from young produced from ova fertilized by the *X. variatus* male, no young could be assigned to the *X. maculatus* male. Copulation was observed after the second male was introduced. Whether insemination took place cannot be ascertained.

2. A *X. maculatus* female, N-2, was fertilized by a *X. maculatus* male, B-20. On the day the first brood was born contact was made with a *X maculatus,* male, 0–21. In the second brood 28 of the 41 young were of the same phenotype as male 0–21 and distinct from the phenotype of male B-20.

3. A hybrid *X. helleri–X. maculatus* female was kept in constant contact with a male *X. maculatus,* K-20. No other fish were kept with this pair. During 136 days of contact, the female gave birth to four broods of 56 young. Two of the fourth brood of fifteen showed the phenotype of male K-20. A fifth brood of nine contained no young of the same phenotype as male K-20.

4. A similar experiment was set up with a brood sister of the female described in (3) above. This female had previously given girth to two broods of young before being placed with male 0–22. During 107 days of constant contact with male 0–22 the female hybrid gave birth to four broods of 52 young. Three young of the fourth brood showed the phenotype of male 0–22.

5. A third hybrid female was placed in contact with a male *X. maculatus*, W-1, after having been inseminated by a male hybrid from the same brood. During 116 days of contact, the female had given birth to four broods. None showed the phenotype of male W-1. The male died before the fifth brood of seven young was born. None of this brood had the phenotype of male W-1.

These experiments, although incomplete and lacking reciprocal crosses, show that once a female has been fertilized by a male of either the same species or a different species, the spermatozoa of the second male can fertilize ova while the spermatozoa of the first male are still viable and fertilizing ova which are in the same broods. These experiments also give an indication of the degree of competition that exists among viable spermatozoa within the ovaries of female viviparous fishes.

A pair of *X. maculatus* was kept in a small aquarium isolated from other fishes to serve as controls and to establish what degree of relationship, if any, existed between constant insemination and the number of young and interval between broods. These results are listed in Table I for female 0–3. At autopsy 42 days after the birth of the last brood this female contained 33 large ova of a very pale, almost transparent color. Usually ova at autopsy are deep amber in color. After development begins the deep amber ova remain the same color and do not change until the embryos are evacuated from the female's body. If any of the yolk material remains at birth, it soon changes to an opaque yellow. No autopsy revealed embryos developing from ova of the pale, transparent type.

Group III

The normal procedure for mating fish in all these experiments was to place the female in a small aquarium which had been occupied for at least a day by an isolated male. In most cases the courtship began almost immediately or as soon as the male was aware of the female in the aquarium. Copulation usually took place after twenty minutes or less of courtship. One lasting contact was considered as sufficient, and an effort was made to limit the mating to only one contact. In some cases females never produced young, although copulation with a lasting contact was observed. Females C-8, N-1, N-4, P-2 and P-4 had contact with only one male but never produced young from these matings. Female C-8 was later mated with another male, C-23, and produced broods from this mating. Females Ca-1, Ck-1, K-4 and P-1 had contact with two males in rapid succession. This was an attempt to have spermatozoa from two different males introduced into the genital tract of the female at as nearly the same time as possible. In two cases, females Ck-1 and P-1, copulation with the second male was accomplished within two minutes after copulation with the first male. Female Ck-1 was later mated with another male, L-20, and produced broods of young.

Whether this failure to produce broods of young can be attributed to lack of insemination and subsequent fertilization is open to question because other factors such

as immaturity may have been responsible. It is noteworthy to mention here that all four attempts to inseminate females with the spermatozoa of two males met with failure. In the total of nine cases of failure in insemination seven males were used (males R-10, B-20, O-21, V-10, C-23, S-10 and X-15). In other matings males R-10, B-20, O-21, V-10, C-23 and X-15 were shown to have produced viable spermatozoa. Male S-10 died before being mated again. Autopsy of female Ca-1 showed that the ovary contained 41 large, deep amber ova. The remainder of this group of females is being used in other experiments and cannot be autopsied at this time. Attempts at hybrid matings of female *X. maculatus* and male *X. helleri* met with failure; no copulation was observed.

Discussion

An attempt has been made, by means of controlled mating, to determine what some of the factors are that affect reproduction in *X. maculatus*. It has been shown by Turner (1937, 1940), Hopper (1943), Wolf (1931) and others that a definite ovarian cycle exists in the female of this species. The cycle may be briefly outlined as follows:

1. Upon completion of development within the follicles of the ovary, embryos are evacuated to the ovarian cavity from which they descend the short genital tract (oviduct) to the exterior of the female's body.
2. As this brood is developing within the ovary, a group of ova which is approximately equal in number to the embryos is becoming larger, accumulating yolk material and approaching a state in which fertilization is possible.
3. A few days after the brood is born the ova are fertilized in the ovarian follicles by the spermatozoa within the ovary. Winge (1922) shows a photomicrograph of spermatozoa lying ready in the ovary of a *Lebistes* female. The heads of the spermatozoa are as near the immature egg as possible.
4. A third group of ova begins to enlarge and the cycle is continued. Bellamy (1924) states that female *X. maculatus* are capable of producing as many as ten broods.

To my knowledge no female *X. maculatus* that has not been inseminated by a male of the same or closely allied species has given birth to young. Hubbs and Hubbs (1946) report an interesting case of *Mollienisia formosa* in which no males of the species are known except from experiments of masculinizing females with gonadotrophic hormones. The female of the species has never been known to reproduce parthenogenically but must first mate with a male of another species before giving birth to young. The young show no signs of paternal inheritance. Hubbs and Hubbs regard this as a species permanently fixed diploid requiring only the stimulus of spermatozoa to initiate development.

That a competition and selection exist among the spermatozoa within the ovary and genital tract of the female is shown in the experimental data for Group II-B. In these experiments various hybrid matings were attempted. These are as follows: (1) a female *X. maculatus* with a male *X. variatus* and male *X. maculatus*, (2) a female *X. maculatus* with two male *X. maculatus* of different phenotypes, (3) three female *X. helleri–X. maculatus* hybrids with three *X. maculatus* males of different phenotypes.

In the first mating the *X. maculatus* male never successfully fertilized any ova of two broods totaling 59 in number, while the male *X. variatus* fertilized ova producing 117 young. The *X. variatus* male was in contact with the female only long enough to effect one insemination; the *X. maculatus* male was placed with the female 92 days after insemination by the *X. variatus* male and four days after the birth of the second brood. This male remained in constant contact with the female for 55 days until the birth of the fourth brood. If the *X. maculatus* spermatozoa had fertilized any of the 59 ova of the third and fourth broods, the young would have shown the crescent tail pattern which was carried by the male. Gordon (1947) has shown that a series of seven dominant autosomal allelic genes controls the pattern of peduncular and caudal pigmentation in *X. maculatus*. Throughout these experiments these data have been used to establish identity and paternal relationship.

All the surviving fish of the first brood produced from this *X. maculatus–X. variatus* cross have differentiated as males. There are differences in the sex-determining mechanisms of the two species. In *X. variatus* the female is the homogametic sex; in domestic *X. maculatus* the male is the homogametic sex. Gordon (1944) confirms the findings of Bellamy (1936) and Kosswig (1935) that all the hybrids from matings of domestic male *X. maculatus* and female *X. variatus* are males. The hybrid mating described in these experiments uses the two heterogametic sexes. The fact that only ten of the twenty young have survived to sexual maturity suggests that other factors may be involved. Perhaps chance has produced such a sex ratio. None of the young from the subsequent broods have reached sexual maturity, and the small number of young in the first brood does not justify any conclusion.

In the second mating described the first male was in contact with the female for a period of 26 days. Forty-two days after the female was introduced into the aquarium containing the male the first brood was born. Beginning on this day the female was placed in contact with the second male for a period of 29 days. On the following day the second brood of 41 was born. Twenty-eight of this brood had the peduncular pattern of the second male; thirteen resembled the first male. The spermatozoa of the second male succeeded in fertilizing the majority of the ova of the second brood. Reference to the other matings in which only one male was used leads to the thought that without intervention of the spermatozoa of the second male, the spermatozoa of the first male would have fertilized the majority of all the ova of the second brood. This indicates that a competition exists among spermatozoa within the ovary or genital tract of the female. The subsequent selection, which is a product of this competition, might have its foundations in the different ages or different quantities of spermatozoa, differential placement within the genital tract, or more subtle differences in viability, size or other factors.

In those experiments in which hybrid females were used, an attempt was made to ascertain the degree of competition between spermatozoa of different species. These females were of a strain that has been bred in commercial hatcheries to include the *X. maculatus* gene for red body color. Gordon (1946) has traced the development of a similar strain which has been developed to include the *X. maculatus* gene for the comet tail pattern in the cytoplasm of the swordtail, *X. helleri*. The red swordtail-platyfish hybrids have been bred by back crosses to the wild-type swordtail to produce a fish that is identical in body form and size, behavior and taxonomic

characters of dorsal fin ray count and lateral line scale count with the wild-type swordtail. In these experiments the females used had previously been inseminated by a male of their own type (in reality a brood brother). They were then placed with *X. maculatus* males. In two of these matings hybrid young carrying the color patterns of the *X. maculatus* males were produced. The third mating was unsuccessful in producing any hybrid young. In one case two hybrids were produced after 136 days of contact; in the other three hybrids were produced after 137 days of constant contact. These periods of contact before hybrid young were produced compared favorably with the periods of 97 to 145 days for the production of young by females in isolation (Groups I-A and I-B). In light of these data there is an indication that the spermatozoa of the *X. maculatus* male are selected against, and that they can fertilize ova only when the spermatozoa of the *X. helleri* male are partially depleted or reduced in number below a critical point.

Another factor which should be considered here is the difference between the courtship and mating behavior of these two species. Clark *et al.* (1948) after a study of their behavior concluded that differences between these fishes do exist and that such psychological barriers can effectively prevent their hybridization in natural situations.

These experiments on the effect of spermatozoa viability, competition and selection have certain general implications as they stand, regardless of the fact that the analyses of the underlying mechanisms are incomplete or entirely wanting. These may be summarized briefly as: (1) offering suggestions concerning the regulation of the reproduction of *X. maculatus* in natural habitats; (2) pointing out a barrier that exists in nature between two sympatric species of livebearing Cyprinodontes; (3) supporting the concept that there are simple social factors such as competition and selection, in operation below the level usually regarded as social, and that these factors can operate in small and subtle ways.

Summary

1. Experiments have been conducted which show that *Xiphophorus maculatus* females which have been successfully inseminated can continue to give birth to young after isolation from contact with male fishes. Although no maximum limit has been established, three to five broods of young has been shown to be the general trend.

2. Females ceased to produce young because of the lack of viable spermatozoa within the ovary. After the spermatozoa within the ovary cease to fertilize ova, the female can be inseminated again and can again produce broods of embryos.

3. By hybrid matings support has been given to the concept of isolating factors between *X. helleri* and *X. maculatus* in nature. Where attempts have been made to inseminate female *X. helleri* with *X. maculatus* spermatozoa, the results were very poor or failed completely.

4. The period of time between insemination and birth of the first brood is longer than the periods of time between subsequent broods produced from the same insemination.

5. Copulation by a female with two male fish in rapid succession failed to produce broods of embryos in these experiments.

6. The general implications of these experiments are suggested.

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TEMPORAL VARIATIONS IN HISTOLOGICAL APPEARANCE OF THYROID AND PITUITARY OF SALAMANDERS TREATED WITH THYROID INHIBITORS[1]

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The fact that thiourea and a number of related chemical compounds are capable of interfering in some way with the normal activity of the vertebrate thyroid gland is now well known. Administration of these substances to common laboratory mammals causes an almost immediate decrease in the thyroid hormone level as evidenced by a lowered rate of metabolism and other symptoms of hypothyroidism. With continued treatment the thyroid gland becomes hypertrophic and hyperplastic, presumably because the decreased content of thyroid hormone in the blood induces a compensatory over-production of thyrotrophic hormone by the pituitary gland. The thyroids of treated animals may thus become markedly enlarged and these thyroid-inhibiting drugs are therefore often referred to as goitrogens.

An extensive literature is available concerning the effects of various goitrogens upon the morphology and physiology of the thyroid gland and the possible mechanism of action of the drugs in laboratory mammals and in the human subject. (See reviews of Charipper and Gordon, 1947; Trotter, 1949; Astwood, 1949; Pitt-Rivers, 1950; Lever, 1951.) Relatively little work has been done on the effects of these compounds on lower vertebrates. However, there is sufficient information available to indicate that they are effective to greater or lesser degree in all types of vertebrates and probably act in essentially the same way in all (Lynn and Wachowski, 1951). Among amphibians the effectiveness of thiourea and related compounds is readily shown by their ability to inhibit metamorphosis in the tadpole (Gordon, Goldsmith and Charipper, 1943; Hughes and Astwood, 1944; Lynn and De Marie, 1946; Koch, 1948; Harms, 1949; Delsol, 1948). However, study of the thyroid glands of treated tadpoles has revealed that although the histological changes observed are similar to those reported for mammals, they are less marked, and after long-continued treatment a decrease in thyroid size and activity may be observed (Gordon, Goldsmith and Charipper, 1945). This decrease also occurs in the adult frog, and Joel, D'Angelo and Charipper (1949) found that after such "regression" the gland may be reactivated by administration of thyrotrophic hormone. It thus appears that the indications of decreased activity observed in the thyroid after long-continued treatment with goitrogens are due to the exhaustion or the impairment of the pituitary thyrotrophic function. Adams (1946) reported that adult salamanders (*Triturus viridescens*) exhibit relatively slight thyroid hyper-

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plasia after long-continued treatment with thiourea. It has been suggested (Lynn and Wachowski, 1951) that this may also be due to a "regression" similar to that noted in the frog.

The present investigation is a study of the histology of both the thyroid and pituitary glands in salamanders treated for periods varying from 5 to 90 days with several different concentrations of four different goitrogens. This study was undertaken in an attempt to ascertain more precisely the effects of these various treatments upon the thyroid, and to find out whether there are any histological changes in the pituitary which can be correlated with the thyroid response.

Materials and Methods

The salamanders used in the present experiments were adult specimens of *Desmognathus fuscus brimleyorum* Stejneger obtained from Dr. Charles Burt of Topeka, Kansas. The animals were kept under laboratory conditions for about a month before use. During this time they exhibited normal activity and a very low mortality rate.

Treatment with thyroid-inhibitors was carried out simply by maintaining the specimens in culture solutions consisting of the appropriate concentrations of the drugs in tap-water. The animals were kept in large finger bowls containing one liter of solution, with seven or eight animals to a bowl. The solutions were changed weekly. The salamanders were fed earth-worms about once a month. At regular intervals, control and experimental animals were removed from the solutions and anesthetized with MS 222. From these animals thyroids and pituitaries were removed. The thyroid glands were fixed in Bouin's fluid and the pituitaries in Zenker's fluid. After two hours fixation, which proved to be sufficient, the material was washed in running water, dehydrated in Cellusolve, cleared in oil of wintergreen and embedded in paraffin. Thyroids were sectioned at 8 μ and stained with Mallory's triple stain. Pituitaries were sectioned at 5 μ and stained according to the technique of Pearse (1950).

In addition to several sets of controls kept in tap-water, the following concentrations of goitrogens were used: 0.005%, 0.01%, 0.05%, 0.1%, 0.3%, 0.5% and 1% thiourea; 0.005%, and 0.01% phenylthiourea; 0.05% allylthiourea; 0.05% thiouracil.

Results

A. Effect of treatment with goitrogenic agents upon the histology of the thyroid gland

All of the treatments used in the present study were effective in producing well-defined histological changes in the thyroid gland of the salamander. However, as one would expect, the effects observed and the time required for their appearance varied with the different drugs and with different concentrations of the same drug. These effects are similar to those recorded in the literature for other vertebrates such as the rabbit (Baumann, Metzger and Marine, 1944) and the chick (Mixner, Reineke and Turner, 1944) and are in particularly close agreement with those reported for other amphibia (Gordon, Goldsmith and Charipper, 1943; Adams, 1946; Gasche and Druey, 1946; Blakstad, 1949; Lynn, 1948; Joel, D'Angelo and Charipper, 1949).

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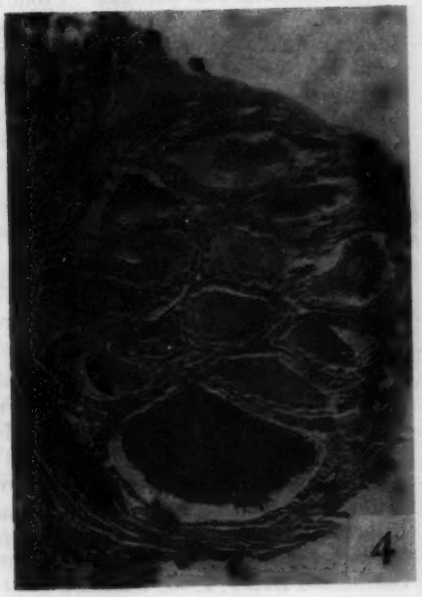
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The general sequence of changes observed in the thyroid following treatment with a goitrogen may be briefly outlined as follows. The first indication of an effect is usually seen in an increased vascularity of the gland. This is often grossly discernible in the intact thyroid and is readily detectable in sectioned material where the hyperemia is indicated by enlarged vessels and an increased number of blood cells (Fig. 2). This change is usually followed in a short time by the appearance of chromophobe droplets ("vacuoles") within the epithelial cells and around the periphery of the colloid mass (Fig. 2). These intracellular and intrafollicular vacuoles are generally considered as reliable indications of increased activity in the gland and they continue to increase in size and number with time. As the vacuolization progresses, the form of the follicular epithelium gradually changes from flat or cuboidal to high columnar and at the same time the staining reaction of the intrafollicular colloid undergoes an alteration from acidophilic to basophilic. Following this the amount of intrafollicular colloid may be greatly decreased so that many follicles have their lumina completely obliterated (Fig. 3), the walls of the follicles being extremely thick and often much folded. Hyperplasia may occur, as indicated by an increase in the number of mitotic figures observed in the epithelium and the whole gland may become grossly hypertrophied. However, such hypertrophy was never so marked as that which is produced by comparable treatment in mammals or even in larval amphibians. After long-continued treatment with relatively high concentrations of goitrogens the appearance of the tissue may return almost to normal with decrease in the height of the epithelium and renewed storage of acidophilic intrafollicular colloid (Fig. 4). The basis for this "regression" will be discussed at length later.

It will be remembered that in these experiments a rather wide range of concentrations of thiourea was investigated while only a few concentrations of three other goitrogens were employed. Using as a basis the foregoing general account of the effects of these substances upon the thyroid, the detailed results of each of the experimental treatments will now be considered.

1. 0.005% thiourea

Thyroids of animals raised in this concentration of thiourea showed no differences from control glands (Fig. 1), until 15 days after the beginning of treatment. At this time there was a definite increase in vascularity and a few intracellular and intrafollicular vacuoles were apparent in some but not all follicles of the experimental animals. By the 25th day of treatment the staining reaction of the colloid mass was basophilic but the follicular epithelium was still cuboidal and the number of vacuoles had not significantly increased. Specimens killed at later periods up to the 88th day of treatment, when the experiment was terminated, exhibited essentially this same condition except for a slight increase in the number of intrafollicular

FIGURE 1. Photomicrograph of normal thyroid gland in *Desmognathus fuscus brimleyorum*. × 150.

FIGURE 2. Photomicrograph of a thyroid gland from an animal treated for 25 days with 0.05% thiouracil. × 280.

FIGURE 3. Photomicrograph of a thyroid gland from an animal treated for 25 days with 0.05% thiourea. × 280.

FIGURE 4. Photomicrograph of a thyroid gland from an animal treated for 85 days with 0.05% thiouracil. × 150.

vacuoles. In none of the specimens studied did the epithelium become columnar nor was there any indication of a decrease in colloid volume.

2. 0.01% thiourea

Clear-cut effects appeared in this series within 10 days after the beginning of treatment. At this time there was marked hyperemia, the follicular epithelium had become low-columnar, both intrafollicular and intracellular vacuoles were abundant and in most follicles the colloid mass, while still acidophilic at the center, showed a wide peripheral zone of basophilic material. These features persisted in the later specimens but the amount of colloid in the gland gradually decreased so that by the 37th day of treatment many follicles appeared to be completely empty. By 72 days the epithelium had become extremely high and the colloid content of the gland was even further diminished. This condition still existed on the 90th day when the experiment ended (Fig. 3).

3. 0.05% thiourea

The effects of this treatment corresponded with those obtained with 0.01% thiourea during the early phases of the experiment. At 10 days the epithelial height was only moderately increased but vacuolization, increased vascularity and basophilia in the colloid were clearly evident. The epithelium became tall columnar by the 15th day, however, and by the 25th day the amount of stored colloid in the gland had been markedly reduced (Fig. 3). This represents the maximum effect obtained with this concentration and the histological condition presented by the thyroid remained unchanged throughout the remainder of the experimental period (72 days).

4. 0.1% thiourea

Specimens exposed to this concentration for 10 days showed striking effects which included not only hyperemia and increased vacuolization but also hypertrophy of the epithelium and decrease in colloid volume. The latter had already proceeded to such a degree that many follicles were completely collapsed. This condition persisted through the 54th day of treatment but in the thyroids of animals killed at later periods there were some indications of a decrease in activity of the thyroid. These indications consisted in some slight reduction in epithelial height and a gradual but rather marked increase in the stored colloid volume. In the specimens tested longest (85 days) some acidophilic colloid was present.

5. 0.3% thiourea

As in the immediately preceding series the earliest specimen studied (10 days) already showed what might be considered almost maximal effects. Material treated for longer periods also corresponded with that seen in the specimens treated with 0.1% thiourea so that this series shows no significant difference in the effectiveness of these two concentrations.

6. 0.5% thiourea

Although animals treated with 0.1% and 0.3% showed marked histological changes in the thyroid within 10 days, specimens treated with 0.5% thiourea did not show such effects. Even up to 25 days of treatment the thyroids of these animals differed from those of the controls only in displaying a slight hyperemia, low columnar epithelium and a moderate degree of intrafollicular vacuolization. By the 37th day the vacuolization had greatly increased and some follicles had a depleted colloid content. However, this seems to have been the period of maxi-

mum effect for in specimens examined after the 37th day the colloid content was gradually increased, the epithelium decreased in height and by the 72nd day of treatment the gland had almost regained the appearance seen in the controls.

7. 1.0% thiourea

The effects of this, the highest concentration of thiourea used, were similar to those obtained with 0.5% thiourea. No significant changes occurred until the 25th day. These increased to a maximum at about the 54th day and then there was a well-defined "regression." However, the decrease in activity had not proceeded so far as to produce a gland of normal appearance when the experiment was terminated at 90 days.

8. 0.005% phenylthiourea

This concentration of phenylthiourea proved more effective than the same concentration of thiourea. Animals treated for 10 days exhibited numerous intracellular and intrafollicular vacuoles and marked hyperemia of the gland. By the 15th day the epithelium had become extremely high and depletion of the colloid content was already far advanced. This same condition was presented by the glands of animals treated up to 54 days but following this a well-defined decrease in activity occurred which was more striking than that seen in any of the thiourea treatments. The height of the epithelium gradually decreased until by 72 days all follicles were lined by flattened cells. Intracellular and intrafollicular vacuoles entirely disappeared and the colloid became dense, homogeneous and completely acidophilic. The gland of the experimental animal at this time thus gave the appearance of a much more inactive thyroid than is seen in the average control.

9. 0.01% phenylthiourea

The effects with this concentration were even more striking than those obtained with 0.005% phenylthiourea. After only 10 days treatment much of the stored colloid had disappeared and by the 25th day there was almost no colloid present in any of the follicles. Specimens given longer treatment show evidence of "regression" although this is not so marked as in the immediately preceding series.

10. 0.05% phenylthiourea

This concentration represents the maximum solubility for this drug and it proved toxic to these animals in a short period. The thyroid of one specimen which survived for 5 days in this concentration was available for study. It is of interest since it already showed increased epithelium height and extreme vacuolization and thus furnished some indication that this concentration is probably even more effective than the preceding.

11. 0.05% thiouracil

This treatment gave no significant results until the 25th day. At this time the epithelium height had increased, intrafollicular vacuoles were abundant and the gland had become hyperemic (Fig. 2). The effects became more pronounced at later stages but by the 85th day there was clear evidence of decreased activity, the epithelium having become low columnar to cuboidal and the colloid having increased in amount (Fig. 4).

12. 0.05% allylthiourea

The effect of this treatment was not apparent until the 37th day but by the 54th day quite marked effects were obtained, the colloid being completely exhausted and the whole gland hypertropic. As in the case of treatment with 0.05% thiouracil,

this was later followed by decreased activity, the epithelium becoming low and the colloid abundant in the later stages studied.

B. Effects of treatment with goitrogenic agents upon the histology of the pituitary

The fact that the thyroid gland is activated by a thyrotrophic principle had well been demonstrated in a number of ways. But precisely which cells of the pituitary are responsible for the elaboration of this hormone is still open to question. One of the aims of the present study was to obtain some evidence on this point.

The generally accepted concept of the mode of action of the goitrogenic substances used in this work is as follows. These drugs interfere in some way with the normal synthesis of the thyroid hormone. As a result of this the thyroid hormone level in the blood is decreased and this decrease stimulates the anterior lobe of the pituitary to produce and/or release its thyroid-activating hormone. The histological changes in the thyroid thus result from stimulation by the thyrotrophic hormone. If the goitrogenic substances are administered continuously, the thyroid, despite its increased cell height and general hypertrophy, continues to be hypofunctional and therefore the pituitary continues to release the thyrotrophic hormone and a goiterous condition results. If a particular cell type in the pituitary is responsible for secretion of the thyrotrophic hormone it would be expected that in animals in which these cells have been over-stimulated by long-continued treatment with goitrogens, some histological changes involving them should be observable. In the material under investigation there is also the possibility that in those animals in which later decreased activity of the thyroid was observed, some histological evidence of the basis for this decrease might be seen in the pituitary. It has been suggested (Joel, D'Angelo and Charipper, 1949) that the thyroid "regression" comes about because the thyrotrophic hormone-secreting cells of the pituitary, after long-continued over-activity, become exhausted and fail to secrete any longer. This would result in a reduction in the thyroid-activating principle in the blood and a consequent return of the thyroid to a normal, or more probably, to an extremely inactive condition.

Although both the pituitary and thyroid glands of all animals were studied, detailed consideration of the histology of the pituitary will be limited to points which bear upon the question of secretory specificity of the cells of the anterior lobe. It is obvious that the most important material to be considered will be pituitaries from animals whose thyroids showed maximum hypertrophic effects and pituitaries from animals which showed the most clear-cut evidence of decreased thyroid activity after long-continued treatment. These, along with pituitaries of control animals, form the basis for the account which follows.

The general appearance of the anterior lobe of the pituitary in the salamander, *Desmognathus fuscus brimleyorum*, with the staining technique employed here is as follows. The acidophilic elements show a clear yellow-staining cytoplasm with a relatively large centrally located nucleus; the basophilic elements exhibit a somewhat excentric nucleus and a cytoplasm which is heavily granulated, and magenta-staining; the chromophobic elements, which are difficult to identify in many cases, show a pale cytoplasmic background and a heavily staining nucleus. The nucleus stains dark blue in all three types of cells.

The first indication of changes in the pituitary were found in animals whose thyroids already exhibited well-marked signs of inhibition. Study of a series of animals in which the thyroids showed progressive activation revealed that the pituitary glands were undergoing concurrent changes characterized by a gradual increase in the proportion of basophilic elements and a decrease in the relative numbers of acidophilic elements. At the same time the chromophobic elements exhibited an increased granulation which may possibly represent transition toward a basophil type.

A study of the pituitaries of specimens whose thyroids exhibited decreased activity after long-continued treatment revealed that the basophilic elements were still predominant but there was evidence of some degenerative change in these cells. The cell outlines tended to be indistinct, the cytoplasm stained less uniformly and many cells had distorted, seemingly pycnotic, nuclei. There was no evidence in this material of any increase in the relative numbers of acidophilic elements. These results tend to support the concept that the decreased activity of the thyroid after long-continued treatment with goitrogens is not due to any return of the pituitary to normality but rather to an abnormality of the basophil cells of the anterior lobe probably resulting from their over-activity and consequent exhaustion.

Discussion

As has been noted, all of the treatments used in the present study produced well-defined histological changes in the thyroid gland but the extent of the changes and the time required for their appearance differed with the treatment. Since thiourea was the only drug for which a wide range of concentrations was tested, conclusions concerning the relation between concentration and goitrogenic effects must be limited to this substance. It will be remembered that treatment with 0.005% thiourea, the lowest concentration tested, was not sufficient to cause maximal activation of the thyroid during the experimental period of approximately three months. With 0.01% thiourea detectable changes in the thyroid appeared sooner and maximal effects were apparent by the 72nd day of treatment. The next higher concentration, 0.05% thiourea, caused an even earlier maximal activation. It appears, however, that the optimal concentration with respect to rapidity of effect lies in the region of 0.1% to 0.3%. These concentrations gave essentially the same results. The thyroids of animals treated for only 10 days appeared to be already fully activated and the condition remained unchanged during the subsequent two months of treatment. During the third month of the experiment the thyroids of animals given these concentrations exhibited signs of a gradual decrease in activity which resulted finally in a histological condition indicating marked inactivity of the gland. The two highest concentrations of thiourea tested, 0.5% and 1.0%, caused less marked changes in the thyroid than did 0.3% or 0.1%. It thus appears that in the present material, the goitrogenic effects of thiourea administered in the culture medium increase with the concentration of the drug up to an optimal concentration at 0.1% to 0.3% and decrease at higher concentrations.

Most of the studies on the effects of thyroid-inhibiting drugs in amphibians have dealt with only one or two concentrations. Bruce and Parkes (1947), however, report that the metamorphosis of larvae of *Discoglossus pictus* is only slightly

delayed when the animals are kept in 0.04% thiourea but is completely inhibited by 0.1% thiourea. Use of 0.2% and 0.5% solutions prevented metamorphosis but also affected the growth of the tadpoles adversely and resulted in a high mortality. Lynn (1948) found that larvae of *Eleutherodactylus ricordii* showed no effects on their growth or metamorphic pattern when treated with 0.001% thiourea, but were increasingly affected by concentrations of 0.005% and 0.05%. The thyroids of the larvae treated with 0.05% thiourea exhibited marked changes but those of animals from the two lower concentrations were not significantly affected. The work of Ratzersdorfer, Gordon and Charipper (1949), although it deals with a lizard rather than an amphibian, is also of interest in this connection for they found that high concentrations of thiourea when administered by injection produced less marked effects on the thyroid than did lower concentrations. These authors ascribed this result to (p. 23) "toxic reactions which tend to mask partially the goitrogenic effect." The nature of the toxic reactions involved is uncertain but it does seem likely that for any goitrogenic drug there is, for any specific animal, an optimal concentration above which less pronounced effects are obtained. This fact may account for the results reported by Adams (1946) who found that the thyroid of adult *Triturus viridescens* which had been kept in thiourea solutions for periods up to 86 days showed relatively slight changes. These newts were in two groups and had been treated with 0.033% and 0.066% thiourea during the first 28 days of the experiment but the concentrations were later increased in two steps so that for the last 44 days the concentrations were 0.528% and 1.056%. In the light of the present results it seems probable that these latter concentrations were above the optimal level and were, therefore, less effective than somewhat lower concentrations would have been.

Of the four different goitrogens tested in the present study, phenylthiourea appears to have the greatest potency. At a concentration of 0.005% this drug produced effects comparable to those obtained with 0.1% thiourea. Thiouracil at a concentration of 0.05% gave effects similar to those resulting from treatment with the same concentration of thiourea while allylthiourea at the same concentration, though its effect was delayed, seemed to produce more marked changes than thiourea. Arrangement of the drugs in decreasing order of effectiveness would thus be: phenylthiourea, allylthiourea, thiourea, thiouracil, with the last two, as far as the present evidence indicates, being approximately equivalent.

Although the relative potencies of a very large number of goitrogens have been tested for common laboratory mammals (Astwood, 1943; Astwood, Bissell and Hughes, 1945) there is very little information available concerning the relative effectiveness of these drugs in cold-blooded animals. Lynn's (1948) results with phenylthiourea and thiourea administered to larvae of the toad *Eleutherodactylus ricordii*, are in agreement with the present results in indicating a greater potency for the former substance. Gasche and Druey (1946) reported that allylthiourea is 10 to 20 times as effective as thiourea in inhibiting metamorphosis in Xenopus larvae and Koch (1948) also found indications of a high potency for allylthiourea in the inhibition of metamorphosis of *Rana temporaria* larvae. Thomas (1947) found that both phenylthiourea and allylthiourea show a greater effectiveness than thiourea when tested on *Rana pipens* tadpoles but he gives no information on the relative potency of the two first-named drugs. Because of differences in the meth-

ods of administration and in the criteria for effectiveness, the results of these studies, so far as they relate to the relative potencies of the goitrogens in question, are difficult to evaluate. It is clear that a detailed assay of the various goitrogens is necessary before any conclusions can be drawn concerning the relative potency of the drugs in amphibians.

The fact that long-continued treatment with thiourea results in decrease in the size and epithelial height of the thyroid was noted in the first investigations of the effects of goitrogen on amphibian material (Gordon, Goldsmith and Charipper, 1943, 1945). These authors found that *Rana pipiens* larvae treated with 0.033% thiourea for three weeks showed slightly enlarged and markedly activated thyroid glands. This condition persisted until about the seventh week of treatment but after this a "regression" occurred which resulted in a decrease in size of the gland as well as a marked change in its histology. Later study by Joel, D'Angelo and Charipper (1949) showed that this same phenomenon occurs in the adult frog after prolonged thiourea treatment and also that the gland can be reactivated by administration of thyrotrophic hormone. These authors, therefore, conclude that the thyroid "regression" is due to a failure in the production or the release mechanism of the thyrotrophic hormone or to a failure in both. It has been seen that in the present experiments a number of the treatments tested, notably those which produced histological evidence of early and maximal thyroid activation, resulted in histological changes indicating decreased thyroid activity after long-continued administration. Thus adult salamanders, like both larval and adult frogs, exhibit this phenomenon.

Since the thyroid activation and the later "regression" are presumed to result from changes in the level of thyrotrophic hormone produced by the pituitary gland, the pituitaries of the experimental animals were subjected to study. It was found that the pituitaries of animals whose thyroids showed well-defined signs of activation differed from those of normal animals in showing an increase in relative numbers of basophilic cells and a decrease in acidophils. In specimens whose thyroids were undergoing "regression" basophils were still predominant but there was evidence that some of these cells were undergoing degenerative changes, the cellular outlines becoming indistinct and the nuclei pycnotic.

There is an extensive literature on the possible localization of secretory function in the various cell types in the pituitary. Most of the evidence concerning the source of the thyrotrophic hormone is derived from studies of the effects of thyroidectomy upon the histology of the pituitary. When the thyroid gland is removed the pituitary responds by an increased production of thyrotrophic hormone and one would assume that the increased activity of the cells which secrete this hormone would be accompanied by some change in their appearance or relative numbers. The results of such investigations have been fully reviewed by Adams (1946) and will not be considered in detail here. While there is some disagreement, most of the work indicates that after thyroidectomy definite changes occur in the basophils. The changes most frequently reported are vacuolization of the cells and an increase in their number. There is considerable divergence of opinion as to whether any significant changes in the acidophils regularly occur.

With the discovery of the goitrogens as tools for carrying out "chemical thyroidectomy," several investigators turned to the use of these drugs as a means

of obtaining further evidence concerning the source of the thyrotrophic hormone. Griesbach (1941) reported that the pituitaries of mammals given a goitrogenic diet exhibit a rapid increase in basophilic elements but that this increase, after reaching a maximum of 56 days, is followed by a return to a more normal condition. The time of return of the pituitary to normal coincided with the time of appearance of colloid in the hyperplastic thyroid glands and Griesbach considered this consistent with the hypothesis that the basophils are the source of the thyrotrophic hormone. Gasche and Druey (1946), who appear to be the only investigators who have previously studied the effect of goitrogens on amphibian pituitaries, refer only briefly to the fact that the pituitaries of *Xenopus laevis* larvae raised in thiourea exhibit the same changes in basophil cells as those which appear after thyroidectomy.

As has been seen, the present work is in agreement with most previous studies in that interference with the normal functioning of the thyroid gland was followed by an increase in the number of basophils in the anterior lobe of the pituitary. The results of long-continued treatment with goitrogens, however, do not agree with those reported by Griesbach. The "regression" which occurred in the thyroid was not accompanied by any return of the pituitary to a normal histological condition; instead, the pituitary showed increasing abnormality with signs of degeneration of some of the basophilic elements. This, however, does not seem surprising since the decreased thyroid activity is assumed to result from failure of the thyrotrophic hormone-producing cells. In fact, since the present results are in such accord with the changes reported by most authors following thyroidectomy, they may be interpreted as adding one more confirmation to the hypothesis that basophils of the anterior lobe of the pituitary are the sources of the thyrotrophic hormone. It must be pointed out, however, that there is another possible interpretation of the pituitary changes. Long-continued treatment with thiourea and similar substances may have direct effects upon the histology of the pituitary and the changes observed therefore may rest upon this basis rather than upon the over-stimulation of thyrotrophic hormone production resulting from thyroid inhibition. So far as the author is aware none of the studies of the results of administration of goitrogens have eliminated the possibility that these drugs might have selective effects upon certain cellular elements in the pituitary which after a time might cause degenerative changes in these elements. It is planned to study the pituitaries of a series of animals given simultaneous treatment with thyroxin and thiourea. Presumably the administration of thyroxin at an appropriate concentration would prevent any stimulation of the pituitary to over-production of thyrotrophic hormone and if any changes in the pituitary then occur such changes could be ascribed to direct effects of the thioura.

Summary

1. A study of the effect of various concentrations of four thyroid-inhibiting substances upon thyroid and pituitary glands in *Desmognathus fuscus brimleyorum* Stejneger has been made.

2. The optimal concentration for thiourea was found to be in the region of 0.01% to 0.3%.

3. The order of effectiveness of the goitrogens tested was phenylthiourea, allylthiourea, thiourea and thiouracil.

4. After long-continued treatment with concentrations of optimal efficiency, the thyroid showed histological evidence of a decrease in activity.

5. Pituitary preparations showed an increase in basophilia as a result of the treatment.

6. Pituitaries of animals which had shown decreased activity in the thyroid preparations exhibited an onset of degeneration in the basophilic elements.

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